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14. ABSTRACT
Prostate cancer is dependent on androgens and the androgen receptor (AR) for disease initiation, maintenance, and progression. Through work by our group and others, it has been shown that there is significant crosstalk between AR and the cell cycle machinery. Most importantly for our study, AR has been shown to induce the G1 to S phase transition in part via regulation of cyclin D1. Cyclin D1 serves as a rheostat to temper the pro-proliferative signaling of AR by directly binding to the receptor and inhibiting it's activity, thus inducing cell cycle arrest. As such, the AR-cyclin D1 crosstalk axis may serve to control the proliferative capacity of prostate cancer cells, and potentially alter the therapeutic efficacy of anti-cancer drugs. The data presented herein will demonstrate that cyclin D1 status does not impinge on the biological outcome *in vitro* of taxane-based therapy.

15. SUBJECT TERMS
androgen receptor, prostate cancer, cyclin D1

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Introduction

Prostate cancer (PCa) is the most frequently diagnosed and second leading cause of cancer death in men in the United States(1). The activity of the androgen receptor (AR) is critical for the development and progression of PCa, and as such is the therapeutic target of disseminated disease(2). AR drives cell cycle progression, at least in part, by regulating mTOR-dependent translation of cyclin D1, which is a critical facet of the G1-S transition machinery(3). Work from our lab and others have determined that cyclin D1 can serve as a negative feedback regulator of AR by directly binding to the receptor and inhibiting its transcriptional activity(4,5,6). The purpose of the proposed studies was to determine the effects of commonly used therapeutics (ionizing radiation, IR; docetaxel, DCTX) on the expression of cyclin D1 protein and subsequent AR regulation, as well as to determine the *in vivo* consequence of disruption of the ability of cyclin D1 to impinge upon AR signaling.

Body

Statement of work

Task 1: Determine the contribution of cyclin D1 down-regulation on response to IR and DCTX. (months 1-12).

A. Determine what residues contribute to cyclin D1 degradation in response to treatment (Months 1-6).

As demonstrated in our first annual report cyclin D1 is degraded in response to genotoxic insult (**Figure 1)**, and threonine 286 is dispensable for cyclin D1 degradation in response to genotoxic stress while leucine 32 is required (**Figure 2)**.

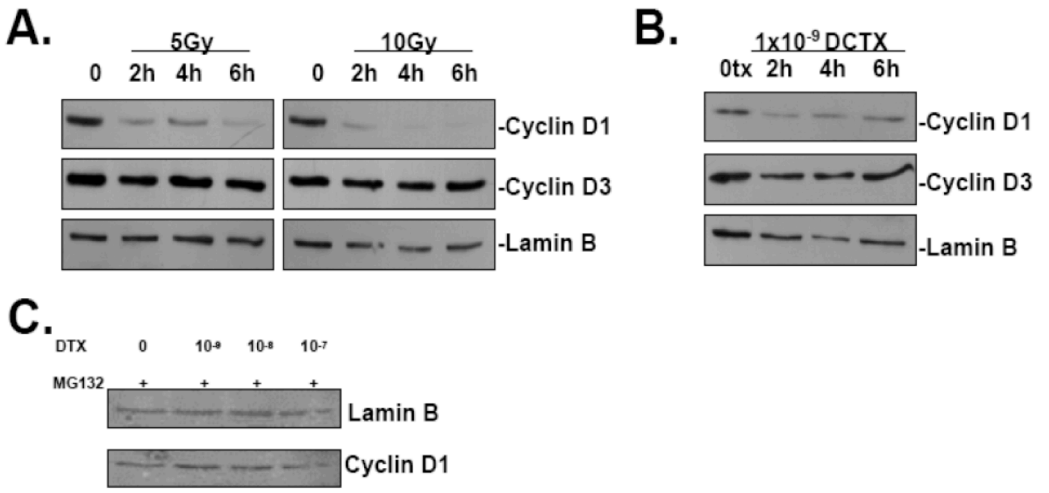


Figure 1. (A) LNCaP cells were treated with the indicated doses of IR and harvested at indicated timepoints, lysed, and total protein was separated by SDS-PAGE, transferred to PVDF, and immunoblotted for indicated proteins. (B) Same as in A, save the cells were treated with indicated dose of DCTX. (C) LNCaP cells were pretreated with proteosome inhibitor (MG132), then treated with indicated doses of DCTX. Cells were harvested 6 hours later, lysed, and total protein was separated by SDS-PAGE, transferred to PVDF, and immunoblotted for indicated proteins.

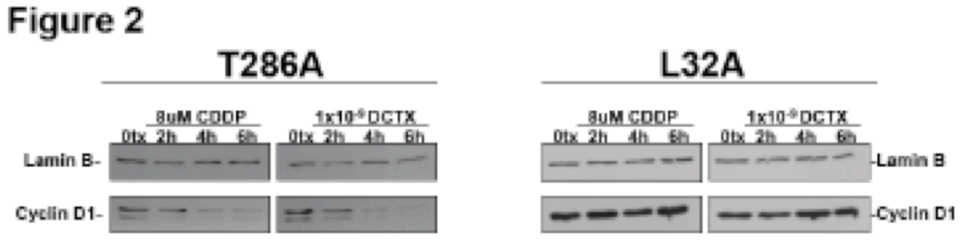


Figure 2. LNCaP cells were transfected with indicated alleles of cyclin D1 (T286A or L32A), treated and harvested as indicated, , lysed, and total protein was separated by SDS-PAGE, transferred to PVDF, and immunoblotted for indicated proteins.

B. Examine the effect of cyclin D1 status on response to therapies with regard to AR activity, cell proliferation/survival
(months 6-12).

As demonstrated in our first annual report, IR and DCTX treatment of PCa cells corresponded with increased levels of prostate specific antigen (PSA) mRNA (**Figure 3A and 3B**), which is a direct AR target gene that is frequently used in the diagnosis and management of PCa. Additionally, it was determined that cells deficient in cyclin D1 as achieved through RNAi technology also corresponds to increased levels of PSA transcript (**Figure 3C**). Data presented in **Figure 4** demonstrate that upon treatment of either LNCaP or C4-2 cells expressing either wild type or mutant (L32A) cyclin D1 with DCTX, there is no discernable difference in cell cycle profiles, regardless of cyclin D1 allele present. Correspondingly, upon knockdown of cyclin D1 in LNCaP cells and subsequent treatment with DCTX, there is no difference in cell number over time, regardless of cyclin D1 status, as shown in **Figure 5**. To examine the comparative effects of the residues of cyclin D1 found to be differentially required for DCTX-induced degration, a similar experiment was performed in both LNCaP and C4-2 cells, and relative cell cycle distribution was determined, as well as cell death as measured by sub-G1 DNA content. As shown in **Figure 6**, DCTX resulted in alterations of cell cycle in cells expressing both alleles. However, there was no statistical difference in the DCTX treated cells regardless of cyclin D1 allele expressed (T286A, which was degraded in response to DCTX, or L32A, which was refractory to DCTX-induced degradation). Together, these data demonstrate that cyclin D1 degradation induced by genotoxic stress does not effect the biological effects in hormone therapy-sensitive (LNCaP) or CRPC (C4-2) cells.

Figure 4

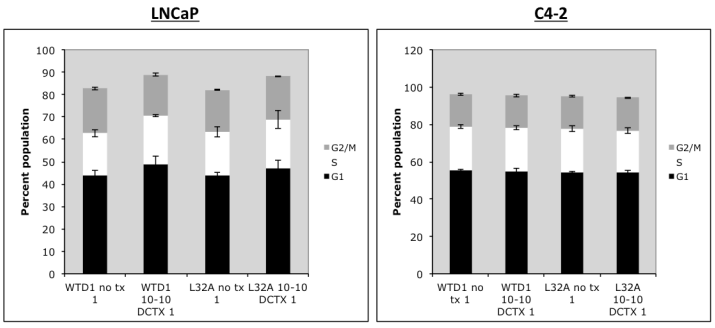


Figure legend: Indicated cell lines were transfected with indicated cyclin D1 alleles. 48 hours after transfection, cells were treated with 1x10^-10 DCTX. 24h later, cells were harvested by trypsinization, fixed, DNA stained with propidium iodide, and prepped for FACS analysis. Data represents at least three independent experiments, mean ± SD.

Figure 5

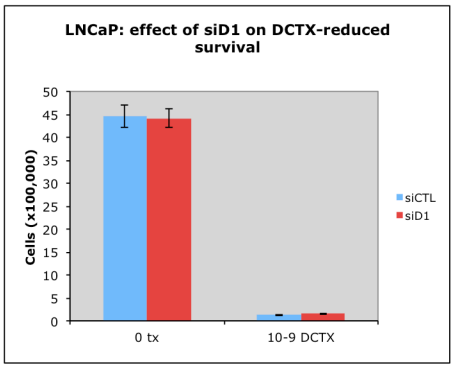


Figure legend: LNCaP cells were seeded at equal density, transfected with either control siRNA, or siRNA targeting cyclin D1. 24h later, the cells were treated with 1nM DCTX, and permitted to grow for 96h, at which time cells were harvested and counting using a hemacytometer and trypan blue exclusion. Data represents at least three independent experiments in triplicate, mean ± SD.

Figure 6

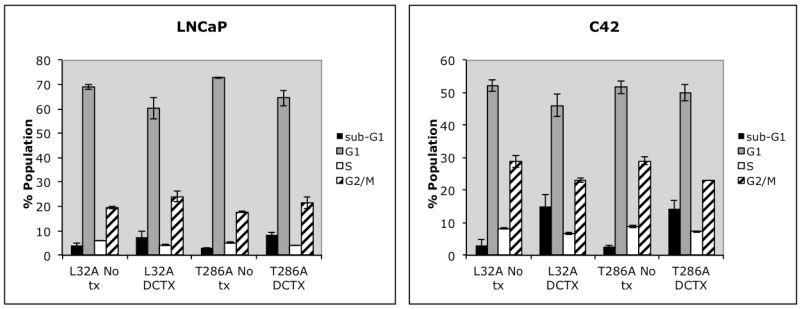


Figure legend: Indicated cell lines were transfected with indicated cyclin D1 alleles. 48 hours after transfection, cells were treated with 1x10^-10 DCTX. 24h later, cells were harvested by trypsinization, fixed, DNA stained with propidium iodide, and prepped for FACS analysis. Data represents at least three independent experiments, mean ± SD.

Task 2: **Dissect the *in vivo* consequence of PCa therapeutics on cyclin D1 and AR (months 18-36).**

A. Examine the expression of degradation-resistant and –proficient alleles of cyclin D1 in xenograft model (months 18-21)

B. Randomize mice in 6 groups: intact/untreated group, intact/IR treated, intact/DCTX treated, castrated/untreated, castrated/IR treated and castrated/DCTX treated. Measure tumor volume with time in mice from untreated groups (months 21-22)

C. Measure tumor volume in treated groups and monitor AR activity (serum PSA) (months 21-22)

D. Excise tumors at the end of the experiment and determine Ki-67 indices, apoptotic indices (TUNEL) and cyclin D1 levels (months 22-36).

As demonstrated in the new data reported under task 1 that cyclin D1 status had no effect on cancer cell biology *in vitro*, these studies were cost-prohibitive and therefore not performed. However, during the funding period two manuscripts were accepted for publication examining therapeutic regimens that alter the cyclin D1 axis.

Study #1: We sought to determine the consequence of theraputics on cell cycle and AR. Attached as **Appendix A** is the manuscript that has been published in Endocrine Related Cancer. The salient points of this manuscript are that targeting the intermediate between AR and cyclin D1, namely mTOR, results in a radiosensitazation of PCa cells that is schedule-dependent. This schedule dependence was determined to be due to relative changes in cell cycle kinetics**.** As shown in **Figure 7**, targeting the mTOR pharmacolocially (mTOR being what controls AR-driven cyclin D1 production) radiosensitizes hormone therapy-sensitive prostate cancer cells in a schedule-dependent manner. This result was confirmed in castration-resistant prostate cancer cells, as shown in **Figure 8.** This schedule-dependent radiosensitization was determined to be reliant on relative cell cycle inhibition, as depicted in **Figure 9.**

Figure 7.

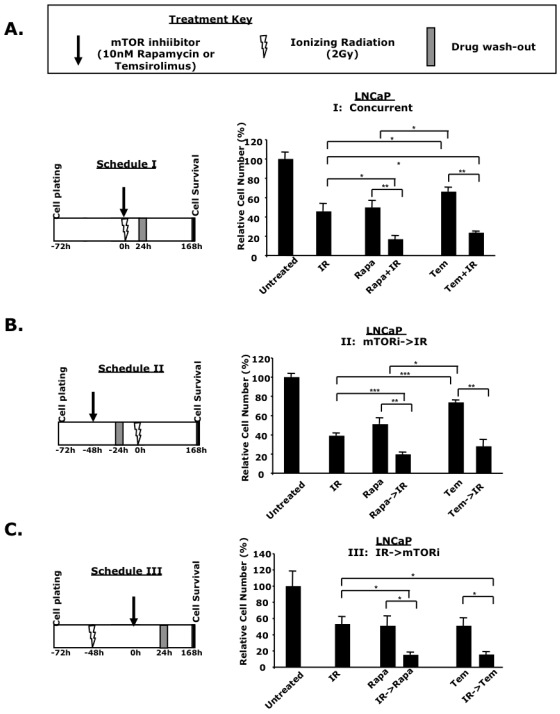


Figure 7. Schedule-specific radiosensitization of PCa cells by mTOR inhibition (A) LEFT PANEL: Schematic of Schedule I treatment strategy (concurrent administration). As depicted, cells were seeded 72h prior to final treatment, mTOR inhibitors, IR, or combination thereof were administered concurrently (Schedule I) (set as time "0"), drug was washed out 24h later, and cell number was assessed 168h after treatment. RIGHT PANEL: LNCaP cells were treated with either 10nM rapamycin (Rapa), 10nM temsirolimus (Tem), 2Gy IR (IR), combination of rapamycin and IR (Rapa + IR), combination of temsirolimus and IR (Tem + IR), or vehicle control (Untreated). Cell survival in the untreated control was set to 100%; averages of three independent experiments and standard deviations are shown. **(B)** Left: Schematic of Schedule II (mTOR inhibitors as neoadjuvant). As depicted, cells were seeded 72h prior to final treatment, administered 10nM of either mTOR inhibitor, which was washed out of culture media 24h later, then 24h after wash, which at time zero was exposure to 2Gy IR, and cell number was assessed 168h post-IR. Right: same as in (A), but with neoadjuvant mTOR inhibitor administration. **(C)** Left: schematic of Schedule III (mTOR inhibitors as an adjuvant post-IR). As shown cells were seeded 72h prior to final treatment, administered 2Gy IR, then treated 48h prior to final treatment (time 0), which in this case was either 10nM of rapamycin or temsirolimus. Right: same as in (A and B), but with adjuvant mTOR inhibitor administration. Statistical analysis of the indicated averages was performed using Student's *t*-test wherein * = p<0.05, ** = p<0.01, and *** = p<0.001.

Figure 8.

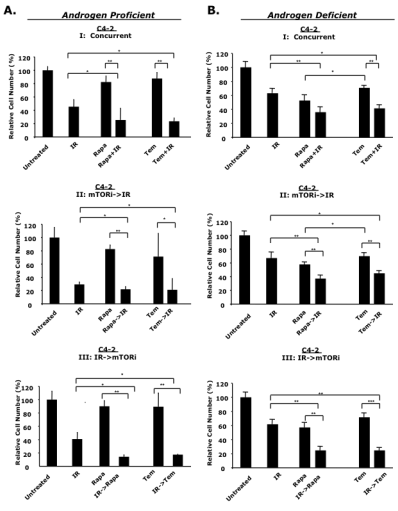


Figure 8. mTOR inhibitors sensitize CRPC cells to effects of irradiation (A) C4-2 cells were treated as in Figure 2. Top: mTOR inhibitor administration concurrent with IR (Schedule I). Middle: mTOR inhibitors administered as neoadjuvant to IR (Schedule II). Bottom: mTOR inhibitors administered as adjuvant to IR (Schedule III). **(B)** C4-2 cells were cultured in steroid-deprived media, and treated as in (A). Cell survival in the untreated control is set to 100%, averages of three independent experiments and standard deviations are shown. Statistical analysis of the indicated averages was performed using Student's *t*-test wherein * = p<0.05, ** = p<0.01, and *** = p<0.001.

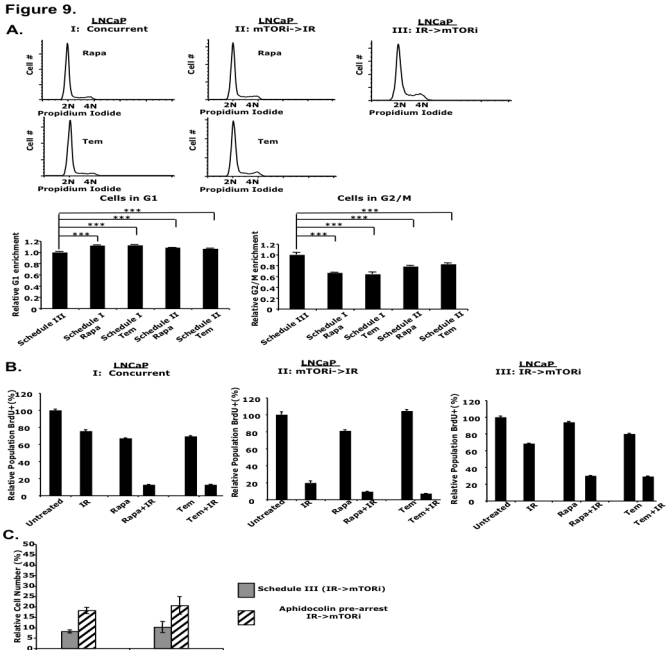


Figure 9. mTOR inhibitor-induced radiosensitization is a function of relative cell cycle-inhibitory effect based on scheduling **(A)** Top: Representative flow cytometry traces for each treatment schedule that LNCaP cells were subjected to as depicted in Fig.2 and harvested just prior to when IR would have been administered, fixed and prepped for FACS analysis of DNA content as described in the Materials and Methods section. Bottom: Quantitation of above. Data represented as relative G1 (left) and G2/M (right) enrichment averages and standard deviations of at least three independent experiments. Schedule III (adjuvant) is set to 1. **(B)** LNCaP cells were treated according to the schemata depicted in Fig. 2, then 24h post treatment, cells were harvested, fixed, and prepped for FACS analysis of BrdU incorporation and DNA content as described in the material and methods section. The data shown depicts the averages and standard deviations of at least three independent experiments analyzing the percent of the cell population that is BrdU positive compared to untreated control, which is set to 100%. **(C)** LNCaP cells were either pre-arrested with vehicle control (grey bars) or aphidocolin (hashed bars) and then subjected to the mTOR inhibitors administered as an adjuvant to IR (Schedule III). Cell number was assessed 168h after the last treatment by trypan blue exclusion and hemacytometer. Graph represents averages and standard deviations of at least three independent experiments, with survival of untreated cells set to 100%.

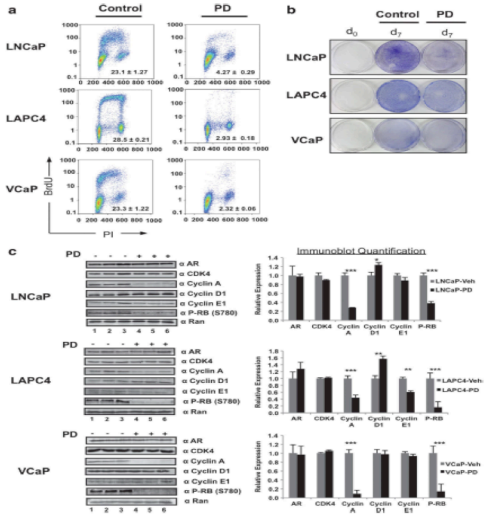


Figure 1. CDK4/6-specific inhibition suppresses proliferation of androgen-dependent PCa cells. The impact of the CDK4/6-specific inhibitor (PD) on proliferation and cell cycle components was characterized in multiple androgen-dependent PCa cell model systems. (a) Bivariate flow cytometry analyses of: LNCaP (upper), LAPC4 (middle) and VCaP (lower) cells treated 24 h with 0.1% dimethylsulphoxide control (left column) or 0.5 μM PD (right column). Profiles are representative of three independent experiments. The x axis denotes relative DNA content as indicated by propidium iodide (PI) staining. The y axis denotes cells undergoing active S-phase as indicated by 2-h pulse-label of BrdU. Inset values: % BrdU incorporation (mean ± s.d., from an experiment performed in biological triplicate). (b) Crystal violet staining at day 7 (d_7) relative to plating at day 0 (d_0) from LNCaP, LAPC4, and VCaP cells initially treated with control or 0.5 μM PD for 24 h. Data are representative of three independent experiments. (c) Immunoblot analyses, from parallel treated cells in panel (a) for the indicated prostate and cell cycle components (left panels) and quantified by LI-COR image analyses (right panels). Loading and quantification are relative to Ran. Grey and black bars = control and PD treatments, respectively. *; **; *** indicates *P*-values: <.05; 0.01; 0.001, respectively.

Study #2: We sought to determine if the kinase partner of cyclin D1 (CDK4/6) could be therapeutically targeted in the first pre-clinical study of CDK4/6 inhibition in PCa. Attached as **Appendix B** is the manuscript that has been published in Oncogene. The

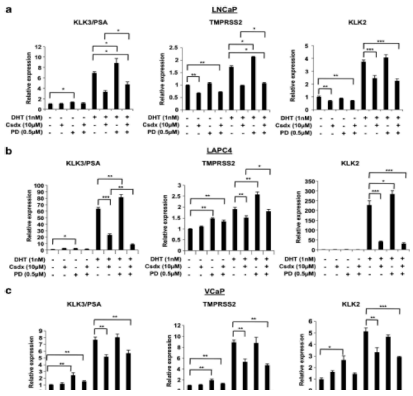


Figure 2. AR-directed therapies are effective in the presence of CDK4/6-specific inhibition. To assess AR activity, androgen-dependent PCa cells: (a) LNCaP (b) LAPC4 and (c) VCaP were cultured 72 h in media containing steroid-deprived serum (5% charcoal-dextran treated (CDT)) then stimulated 24 h with (or without) DHT (1 nM) in the presence of PD (0.5 μM), Csdx (10 μM) or combination of PD and Csdx. Relative mRNA expression normalized to glyceraldehyde 3-phosphate dehydrogenase was determined by quantitative PCR (qPCR) for the known AR-target genes: *KLK3/PSA* (left), *TMPRSS2* (middle) and *KLK2* (right). Indicated treatments for each gene are relative to non-DHT and non-drug treated cells. *; **; *** indicates *P*-values: <0.05; 0.01; 0.001, respectively.

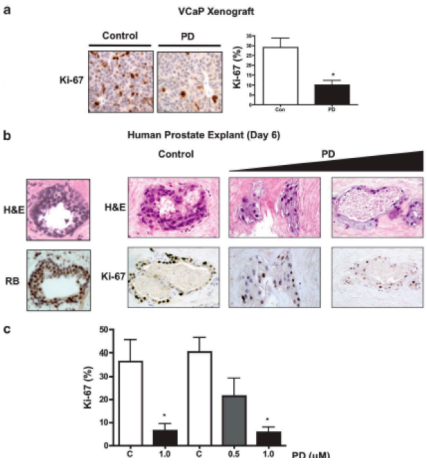


Figure 5. CDK4/6-specific inhibition suppresses proliferation of xenografts *in vivo* and primary human prostate tissue *ex vivo*. The efficacy of PD on PCa was determined by: (a) Proliferative Ki-67 marker analysis from VCaP xenografts, grown in SCID mice, treated with control ($n = 4$ mice) or PD ($n = 4$ mice). Mice were treated with 150 mg/kg of PD, as described detail in the Materials and methods section. Representative images (20 ×) are shown (left panel) and Aperio-based quantification of % Ki-67 (right panel). *Indicates a *P*-value <0.05. (b) Representative images from histo- and immunohistological analyses of human prostate tissues used for *ex vivo* tissue explant culture: left panel, images (20 ×) from hematoxylin and eosin (H&E) and RB-stained tissue before processing for culture; right panel, H&E images (20 ×, upper row) and Ki-67 images (20 ×, lower row) from *ex vivo* explant tissues treated 6 days with control (left column) and 0.5 (middle column) or 1 μM PD (right column). (c) Aperio-based quantification of % Ki-67 from human explant tissues cultured *ex vivo* for a short (day 2) and long (day 6) treatment with control (C) or 0.5 or 1 μM PD. Bars represent (mean ± s.e.m.) from five patient specimens. *Indicates a *P*-value <0.05.

salient points of this manuscript are that CDK4/6 inhibition suppresses proliferation of PCa cells, does not diminish the efficacy of AR-directed therapeutics, and serves to radiosensitize in models of hormone-dependent disease. Additionally, in castration-resistant models, CDK4/6 inhibition diminishes cell proliferation in a manner dependent on expression of the RB tumor suppressor, and that the above results are recapitulated both *in vivo* utilizing xenografts and *ex vivo* utilizing a novel explant assay of primary human cancer tissue. As shown in **Figure 10**, targeting CDK4/6 (the kinase partner of cyclin D1) suppresses prostate cancer cell proliferation. **Figure 11** depicts the effect of this inhibition on AR transcriptional activity, and **Figure 12** demonstrates the effect of blocking cyclin D1 kinase activity *in vivo* and using an *ex vivo* system of prostatectomy specimen culture.

Task 3: Determine the cooperation of the cyclin D1 AR-repressive function (RD) with cytotoxic agents.
(months 6-18)
A. Introduce RD into cell systems of clinical relevance, treat with IR and/or DCTX
(months 6-18)
B. Examine effect on AR activity, cell proliferation/survival
(months 6-18)
Prior to initiation of these studies, we sought to compare full-length cyclin D1 and RD (cyclin D1 repressor domain) with regard to PCa cell model cytotoxicity. As demonstrated in **Figure 7**, while RD results in decreased cell number over time, indicative of cytotoxicity, full length cyclin D1 demonstrates a merely cytostatic phenotype. Future studies will examine the impact of combining RD and genotoxic stress.

Figure 7

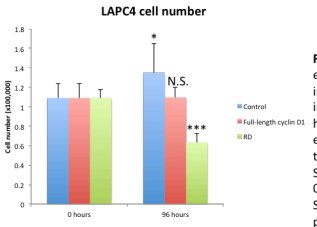
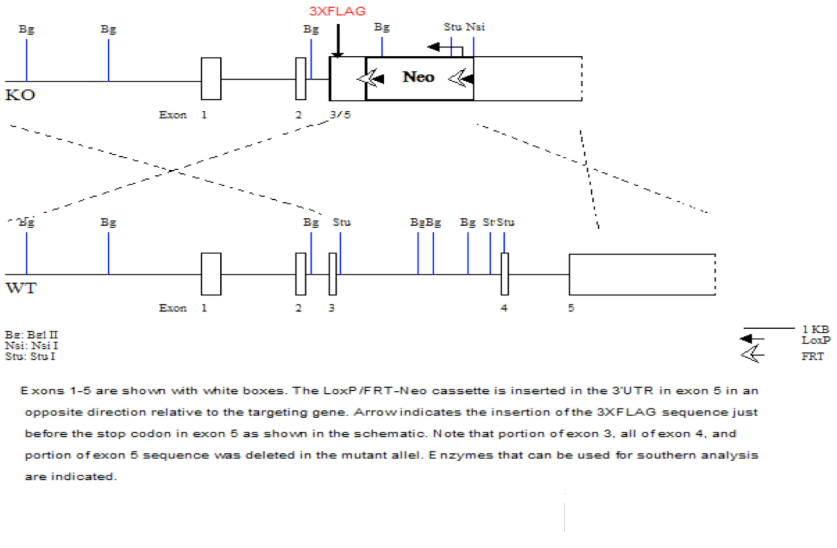


Figure legend: Cells were seeded at equal density, and infected with indicated adenoviruses. 96h after infection, cells were counted using a hemacytometer and trypan blue exclusion. Data represents at least three independent experiments ± SD. Statistical significance between 0h and 96h was determined by Student's t test: *= p<0.05, ***= p<0.001, N.S. = not significant

Task 4: Determine the in vivo consequence of cyclin D1-mediated AR regulation in the prostate
(months 1-36)
A. Generate knock-in mice (3xFLAG-tagged full-length cyclin D1 and cyclin D1-ΔRD).
(months 1-17)
As described in our previous report, these mice have been generated via the scheme in **Figure 8.**
B. Examine the effect of cyclin D1 on AR signaling in the prostate (months 17-36).
As described in our previous reports, these mice have been generated via the scheme in **Figure 8.**



Exons 1-5 are shown with white boxes. The LoxP/FRT-Neo cassette is inserted in the 3'UTR in exon 5 in an opposite direction relative to the targeting gene. Arrow indicates the insertion of the 3XFLAG sequence just before the stop codon in exon 5 as shown in the schematic. Note that portion of exon 3, all of exon 4, and portion of exon 5 sequence was deleted in the mutant allel. Enzymes that can be used for southern analysis are indicated.

Figure 8. Sehmatic representing knock-in strategy for cyclin D1ΔRD allele. Exons 1-5 are shown as white boxes. A LoxP/FRT-Neo cassette is instered in the 3'UTR in exon 5 in the opposite direction relative to the targeting gene. Arrow indicates insertion of the 3xFLAG tag sequence just before the stop codon in exon 5. Note that a portion of exon 3, all of exon 4, and a portion of exon 5 sequence was deleted in the mutant allele. These deleted sequence represent the genomic region responsible for RD coding. Enzymes that can be used for southern analysis are indicated.

Multiple organs demonstrate expression of the 3xFLAG tagged allele as demonstrated by **Figure 9**.

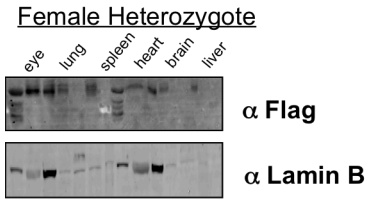


Figure legend: Indicated organs were dissected from a heterozygote female. The tissue were homogenized, lysed, and total protein was separated by SDS-PAGE, transferred to PVDF, and immunoblotted with FLAG antisera (to detect novel cyclin D1 allele) and Lamin B (control)

Currently, we are endeavoring to breed out the ES cell selective Neomycin cassette, and increase our colony number (for male homozygous wild-type and mutant alleles) so as to complete the proposed studies.

Training program

Task1: Didactic coursework and laboratory research

A. Didactic coursework->completed

B. Laboratory research (months 1-36)->completed. Thesis defended on August 20th, 2012

Task 2: Lab meetings

Weekly (months 1-36)->completed

Task 3: Joint lab meetings

Bi-weekly (months 1-36)-> completed

Task 4: Paper of the day

Participate daily. Present bi-weekly (months 1-36) -> completed

Task 5: Prostate Cancer Translational Research Seminar Series

Once monthly (months 1-36) -> completed

Task 6: Journal club

Weekly participation. Presentation once per academic quarter (months 1-36) -> completed

Task 7: Conferences

A. Attend Keystone Symposia on Nuclear Receptors and present research. Completed

B. Attend annual AACR conference and present research. Completed

C. Attend other meeting of prostate cancer relevance and present research (months 12-36) -> completed

Task 8: Other

A. Continued interaction with researchers and clinicians (months 1-36) -> completed

B. Attend seminars at Kimmel Cancer Center at Thomas Jefferson University that occur multiple times per week (months 1-36) -> completed

Key research accomplishments:

-cyclin D1 degradation occurs with rapid kinetics following genotoxic insult
-this degradation is dependent upon the proteasome
-threonine 286 of cyclin D1 is dispensable for genotoxic insult-induced degradation, while leucine 32 is requisite
-loss of cyclin D1 in PCa cells corresponds with increased AR transcriptional activity
-genotoxic induced cyclin D1 degradation does not have an impact on biological effect of genotoxic stress

-ablation of cyclin D1 protein by RNAi does not impact biological outcome of genotoxic challenge
-cyclin D1 status is not a determinant of the effects of docetaxel in LNCaP cell models
-full-length cyclin D1 elicits cytostatic effects, while RD alone elicits cytotoxic effects in PCa cell models
-targeting the intermediate between AR and cyclin D1 therapeutically (mTOR) radiosensitizes PCa cells
-pharmacological targeting of cyclin D1 kinase activity (CDK4/6) elicits cytostatic effects in hormone therapy-sensitive and castration-resistant *in vitro* dependent upon RB expression
-CDK4/6 inhibitors do not counteract AR-directed therapy
-CDK4/6 inhibition radiosensitizes PCa cells
-CDK4/6 inhibition elicits these same effects *in vivo* and *ex vivo*
-full-length cyclin D1 elicits cytostatic effects, while RD alone elicits cytotoxic effects in PCa cell models
-a novel mouse model has been successfully generated that expresses an allele of cyclin D1 predicted to be incompetent for AR inhibition and is being characterized fully

Reportable outcomes

During the funded period, I have completed by PhD in Genetics at Thomas Jefferson University, winning the Jefferson School of Biomedical Sciences Yun Yen, MD, PhD and Sophie Yen Thesis Prize for Distinguished Research in Pathobiology.

I have applied for and been offered a position as a postdoctoral research fellow at the Kimmel Cancer Center at Thomas Jefferson University in the laboratory of Karen E. Knudsen, PhD based on the experience/training supported by this award.

Conclusion

Prostate cancer management remains a serious health concern, and understanding the mechanisms of both therapeutic efficacy and bypass are of great importance. Studies completed this far demonstrate that: 1) cyclin D1 is degraded in response to genotoxic insult in PCa cells dependent upon specific residues and the proteasome; 2) this degradation does not impact biological function of genotoxic stress,despite the observation that there is an apparent increase in AR transcriptional activity; 3) mTOR inhibitors may be viable radiosensitizers in PCa (for which there are currently no approved means of doing so); and 4) pre-clinical data indicate targeting cyclin D1 kinase activity to be worth pursuing in the clinical setting. Studies looking at novel means to target the cyclin D1-AR axis are being aggressively pursued.

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Appendix A

mTOR is a selective effector of the radiation therapy response in androgen receptor-positive prostate cancer

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Abstract

Ionizing radiation (IR) is used frequently in the management of multiple tumor types, including both organ-confined and locally advanced prostate cancer (PCa). Enhancing tumor radiosensitivity could both reduce the amount of radiation required for definitive treatment and improve clinical outcome. Androgen suppression therapy improves clinical outcomes when combined with radiation therapy but is associated with significant acute and chronic toxicities; hence, there is a clear need for alternative means to increase the therapeutic window of radiotherapy. Herein, it is demonstrated that the mammalian target of rapamycin (mTOR) inhibitors rapamycin (sirolimus) and temsirolimus limit both hormone therapy (HT)-sensitive and castration-resistant PCa (CRPC) cell proliferation as single agents and have a profound radiosensitization effect when used in combination with IR. Importantly, the observed radiosensitization was influenced by the treatment schedule, in which adjuvant administration of mTOR inhibitors was most effective in limiting PCa cell population doubling. This schedule-dependent influence on *in vitro* treatment outcome was determined to be the result of relative effects on the cell cycle kinetics. Finally, adjuvant administration of either mTOR inhibitor tested after IR significantly decreased clonogenic cell survival of both HT-sensitive and CRPC cells compared with IR alone. Taken together, these data demonstrate that inhibition of mTOR confers a radiosensitization phenotype that is dependent on relative cell cycle kinetics and provide a foundation for clinical assessment.

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Introduction

Prostate cancer (PCa) is the most frequently diagnosed non-cutaneous malignancy and the second leading cause of death due to cancer in men in the United States (Jemal *et al*. 2010). Treatment options for localized disease include watchful waiting, surgery, and radiotherapy (RT; Klein *et al*. 2009). In the context of definitive treatment, adjuvant therapy after radical prostatectomy, and in some cases metastatic disease, RT is becoming of increasing significance for successful management of PCa (Kwok & Yovino 2010).

Androgens and the cognate receptor (androgen receptor (AR)) have a well-described function in all stages of PCa. If disseminated at the time of diagnosis, first-line therapy is targeted against the AR signaling axis. Suppression of AR activity is achieved by using GnRH agonists that induce ligand depletion (chemical castration) and is sometimes used in combination with direct AR antagonists (such as bicalutamide; Klotz 2006, Loblaw *et al*. 2007, Taplin 2007, Chen *et al*. 2008, Knudsen & Scher 2009). For locally advanced or high-risk disease, RT is frequently used, thus underscoring the need to delineate the impact of combination therapy. AR-directed therapeutics is initially effective due to the dependence of this tumor type on AR signaling; however, after a median time of 2–3 years, tumors recur and are deemed 'castration resistant'

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(castration-resistant PCa (CRPC)). CRPC development is highly attributed to inappropriate resurgence of AR activity, which occurs despite the absence of circulating serum androgens and administration of direct AR antagonists (Knudsen & Scher 2009, Yuan & Balk 2009). Strikingly, few therapeutic options have shown efficacy against this stage of the disease, and a major goal of current translational research is to develop means for preventing or delaying progression to CRPC. One means by which PCa cells bypass AR-directed therapeutics involves upregulation of rapamycin (Rapa)-sensitive signaling (Mousses *et al*. 2001), and that combining mammalian target of rapamycin (mTOR) inhibition with AR-directed therapies prolongs hormone sensitivity in xenograft models of PCa (Schayowitz *et al*. 2010). Moreover, AR is known to promote mTOR activity (Xu *et al*. 2006*b*), thus suggesting that combining mTOR- and AR-directed therapeutics may cooperate to improve cellular and clinical responses to therapy.

Given the poor outcomes associated with resurgent AR activity and CRPC development, it is imperative to develop new means for enhancing therapeutic efficacy and thus to prevent the transition to CRPC. In patients with locally advanced PCa treated with RT alone, the 5-year disease-free survival rate is 40% (Bolla *et al*. 2002). Therefore, improving the overall efficacy of RT could be of significant clinical benefit. Several potential mechanisms lead to RT failure, including altered proliferative and pro-survival potential, both of which are frequently observed in PCa.

A frequent genetic lesion that leads to both events is loss of PTEN function. Sixty percent of PCa demonstrate loss of heterozygosity at the *PTEN* locus (Cairns *et al*. 1997, McMenamin *et al*. 1999). Decreased expression of PTEN has been detected in 85% of primary PCa tumors compared to normal prostatic tissue of the same patient (Kremer *et al*. 2006), and patients with tumors harboring mutant PTEN have decreased survival, higher metastatic frequency, and higher prostate-specific antigen (PSA) levels, suggesting higher AR activity (Pourmand *et al*. 2007); therefore, PTEN is one of the most frequently altered genes in human PCa and is associated with lethal tumor phenotypes. The PTEN phosphatase serves at the molecular level to counteract the functions of phosphoinositide 3-kinase, which promotes proliferation and cell survival, in part through activation of mTOR (Sansal & Sellers 2004). Akt serves as an intermediate signaling molecule for mTOR, which is a serine/threonine kinase that mediates cell growth, proliferation, survival, protein translation, and other oncogenic functions.

mTOR activity is often deregulated in Pca (Kremer *et al*. 2006), in part due to the prevalence of PTEN dysfunction. Genomic deletion of PTEN is associated with both increased Akt activation and AR activity (Sircar *et al*. 2009). mTOR mediates proliferation in PCa cells, at least in part, due to androgen-induced upregulation of D-type cyclin translation (Gao *et al*. 2003, Xu *et al*. 2006*b*). This event is suggested to, therefore, promote cell cycle progression. In addition, mRNA translation events that are dependent on mTOR are rapidly activated in response to ionizing radiation (IR), resulting in DNA repair and survival (Braunstein *et al*. 2009). As such, the mTOR signaling pathway is a potential target for enhancing RT efficacy and improving therapeutic intervention in PCa.

Pharmacological mTOR inhibition has been demonstrated to block the induction of the proliferative, pro-survival, and oncogenic functions of mTOR (Hidalgo & Rowinsky 2000), with remarkable effects in PTEN-deficient tumors. mTOR inhibitors (e.g. everolimus) have been approved by the FDA for treatment of renal cell carcinoma based on a successful phase III clinical trial (Motzer *et al*. 2008); thus, mTOR is an established therapeutic target and mTOR inhibitors appear to be reasonably well tolerated. At the cellular level, mTOR inhibitors have been shown to sensitize multiple tumor types to DNA damage-inducing agents, including IR, using both *in vitro* and *in vivo* models of human disease (Beuvink *et al*. 2005, Wu *et al*. 2005, Cao *et al*. 2006, Aissat *et al*. 2008, Morgan *et al*. 2008, Ekshyyan *et al*. 2009, Fung *et al*. 2009, Matsuzaki *et al*. 2009, Murphy *et al*. 2009, Saunders *et al*. 2010). Moreover, mTOR signaling has been implicated as a determinant of cell survival in response to DNA damage (Shen *et al*. 2007).

This study assessed the impact of mTOR inhibition in clinically relevant models of hormone therapy (HT)-sensitive PCa and CRPC tumor cells both alone and in combination with RT. Survival analyses revealed that mTOR inhibitors sensitized both HT-sensitive PCa and CRPC cells to IR at clinically attainable doses. The impact of sequence of mTOR inhibition as a radiosensitizer was also assessed, where it was observed that the radiosensitization events were influenced by the scheduling. Strikingly, mTOR inhibitors were most effective at conferring radiosensitization effects when administered in the adjuvant setting. Schedule dependence was determined to be due to cell cycle kinetics, in which neoadjuvant use of mTOR inhibitors limited entry of the cells into a state of active DNA replication. On combining these studies, it is demonstrated that mTOR inhibitors radiosensitize AR-positive PCa cells dependent on treatment schedule and relative cell cycle inhibition and provide evidence of a viable combinatorial treatment strategy.

Materials and methods

Cell culture and reagents

LNCaP, C4-2, and LAPC4 cells were cultured under standard conditions at 37 °C and 5% CO_2 as described previously (Sharma *et al*. 2010). Rapa was obtained from Calbiochem (San Diego, CA, USA) and dissolved in DMSO. Temsirolimus (Tem) was obtained from LC Laboratories (Woburn, MA, USA) and dissolved in ethanol.

Ionizing radiation

A Panatek orthovoltage X-ray irradiator was used to deliver IR. The irradiator was calibrated daily using a Victoreen dosimeter.

Cell counting/survival

To monitor cell number over time, indicated cells were seeded on poly-L-lysine-coated dishes at equal densities and subjected to treatment/schedules described. At the time of harvest, cells were trypsinized and counted using Trypan Blue exclusion and a hemacytometer. Total cell number was determined from at least three independent experiments of three biological replicates.

Cell cycle analysis/bivariate FACS

To monitor bromodeoxyuridine (BrdU) incorporation/DNA content, LNCaP cells were seeded on poly-L-lysine-coated dishes at equal densities and subjected to treatment/schedules described. Two hours prior to harvest, cells were incubated with BrdU (1:1000, Amersham Cell Proliferation Labeling Reagent, GE Healthcare, Buckinghamshire, UK). After labeling, cells were trypsinized and harvested, washed with PBS, and then re-suspended in PBS. Cells were then fixed with cold 100% ethanol, pelleted, then re-suspended in 2 M HCl, and incubated for 20 min at ambient temperature. HCl was neutralized with 0.1 M sodium tetraborate, washed with IFA buffer, followed by a wash with IFA buffer supplemented with 0.5% Tween 20, then re-suspended in IFA buffer containing 6% FITC-conjugated anti-BrdU anti-sera (BD Biosciences, San Diego, CA, USA), and incubated for 45 min. Cells were then washed with IFA buffer supplemented with 0.5% Tween 20, stained with propidium iodide (0.2 g/ml), and subjected to flow cytometry. Samples were quantified on a Coulter Epics XL-MCL using XL System II Software (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo Software (Tree Star, Inc., Ashland, OR, USA). To monitor only the DNA content, LNCaP cells were seeded on poly-L-lysine-coated dishes at equal densities and subjected to treatment/schedules described. Cells were trypsinized and harvested, washed with PBS, and then re-suspended in PBS. Cells were then fixed with cold 100% ethanol, pelleted, stained with propidium iodide (0.2 g/ml), and subjected to flow cytometry. Samples were quantified on a Coulter Epics XL-MCL using XL System II Software (Beckman Coulter) and analyzed using FlowJo Software (Tree Star, Inc.).

Clonogenic cell survival

Exponentially growing cells were trypsinized and counted using Trypan Blue exclusion. Cells were diluted serially to appropriate concentrations and plated into 50 ml tissue culture flasks in triplicate for 24 h. Then, cells were treated with increasing doses of IR (0, 2, 4, 6, and 8 Gy). After 24 h, cells were treated with Rapa (10 nM), Tem (10 nM), or nothing. After 14 days of incubation, the colonies were fixed and stained with 4% formaldehyde in PBS containing 0.05% crystal violet. Colonies containing >50 cells were counted. Surviving fraction was calculated as (mean colony counts)/((cells inoculated)$\times$(plating efficiency)), in which plating efficiency was defined as (mean colony counts)/(cells inoculated for un-irradiated controls).

Results

Single-agent mTOR inhibitors or IR limit HT-sensitive PCa cell growth

mTOR activity has been observed to be increased in PCa through various mechanisms and upstream signaling defects. To challenge the consequence of mTOR inhibition in PCa, HT-sensitive cells were treated with increasing doses of two pharmacological inhibitors of mTOR activity, Rapa and Tem. It has been demonstrated that in this cell type, androgens induce mTOR signaling that culminates in cell cycle progression via an increased translation of cyclin D1 (Xu *et al*. 2006*b*), which is part of the molecular machinery responsible for the G_1–S phase transition (Baldin *et al*. 1993). Consistent with previous reports, mTOR inhibition resulted in decreased cell number after 72 h of treatment (Fig. 1A; van der Poel *et al*. 2003). As demonstrated, there was no significant difference between either of the mTOR inhibitors tested with regard to response at any of the doses tested. As IR is used as definitive treatment for localized, HT-sensitive PCa, the effect of IR on HT-sensitive cells was assessed. These results demonstrate a dose-dependent decrease in population cell doubling after exposure to IR (Fig. 1B).

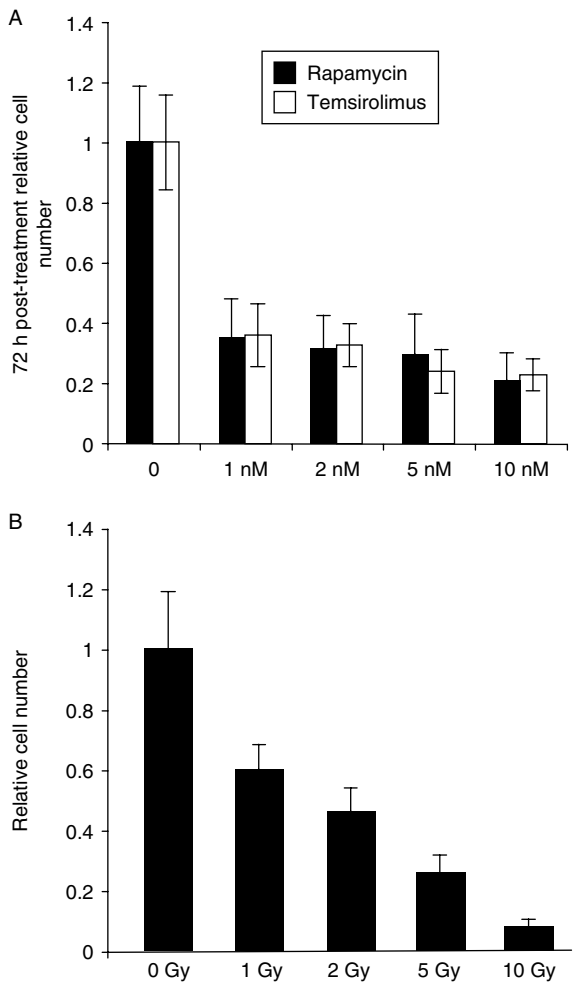

Figure 1 mTOR inhibitors and ionizing radiation (IR) are sufficient to limit hormone therapy-sensitive prostate cancer (PCa) cell doubling as single agents (A). LNCaP cells were treated with indicated doses of rapamycin, temsirolimus, or vehicle control. 72 h after treatment, cell number was assessed via Trypan Blue exclusion using a hemacytometer. Cell number in the vehicle controls was set to '1'. Averages of three independent experiments and S.D. are shown. (B) LNCaP cells were exposed to the indicated doses of IR. 168 h after treatment, cell number was assessed via Trypan Blue exclusion using a hemacytometer. Cell number in un-irradiated controls was set to '1'. Averages of three independent experiments and S.D. are shown.

Taken together, these data demonstrate that single-agent mTOR inhibitors and IR affect HT-sensitive cells. Additionally, there was no observable difference in the efficacy of Rapa and Tem in this context.

Combining mTOR inhibition and IR is more effective than single agent in limiting HT-sensitive PCa cell number

While IR is a frequently used treatment modality for locally advanced disease, there is a 10–60% recurrence rate (Allen *et al*. 2007), suggesting that means to improve the efficacy of RT is a significant clinical need. Based on this premise, and the observation that mTOR signaling is both involved in PCa cell cycle progression/survival (Gao *et al*. 2003, Xu *et al*. 2006*b*) and induced by IR (Tirado *et al*. 2003, Shen *et al*. 2007), the impact of mTOR inhibition on the response to IR was determined in HT-sensitive cells. To determine whether scheduling of the treatment affected outcome, a strategy was used to test concurrent (Schedule I), neo-adjuvant (Schedule II), and adjuvant (Schedule III) mTOR inhibitor administration. The time from final treatment to assessment of outcome was identical for all schedules tested. Cells were sensitized to IR when mTOR inhibition was co-administered (Fig. 2A; compare IR, Rapa, and Tem alone to Rapa + IR and Tem + IR). To assess impact on the neo-adjuvant context, mTOR inhibitors were administered 48 h prior to IR treatment; as shown in Fig. 2B, there was a significant decrease in cell number following this treatment schedule (compare single agents vs combination). Finally, adjuvant mTOR inhibition conferred radiosensitization effects (Fig. 2C). Notably, Schedule III was most effective in limiting cell doubling (~15% of control) when compared with Schedule I (~20%) or Schedule II (~23%), suggesting that scheduling of treatment should be considered in therapeutic design. The impact of schedule was likely attributed to relative effects on cell cycle progression and was conserved in another HT-sensitive PCa cell model (LAPC4; Supplementary Figure 1, see section on supplementary data given at the end of this article). The LAPC4 model maintains wild-type PTEN (Whang *et al*. 1998) and harbors a mutant p53 allele (van Bokhoven *et al*. 2003), suggesting that neither PTEN or p53 status alters the radiosensitization effect of mTOR inhibitors. Although the contribution of PTEN status to mTOR inhibitor sensitivity has been documented, data herein demonstrate that both PTEN-proficient and PTEN-deficient cells can be radiosensitized by mTOR inhibition. Of note, the PTEN-proficient cell line LAPC4 may be intrinsically more radioresistant compared to the other model systems used. This is not without precedent, as it as been demonstrated that this cell line is relatively insensitive to chemotherapy (Xu *et al*. 2006*a*, Qian *et al*. 2010). However, mTOR inhibition still renders this cell type more sensitive to radiation. Taken together, these data demonstrate that combining mTOR inhibitors with IR is effective in limiting PCa cell number over time regardless of scheduling; however, adjuvant use of mTOR inhibitors may be most efficacious.

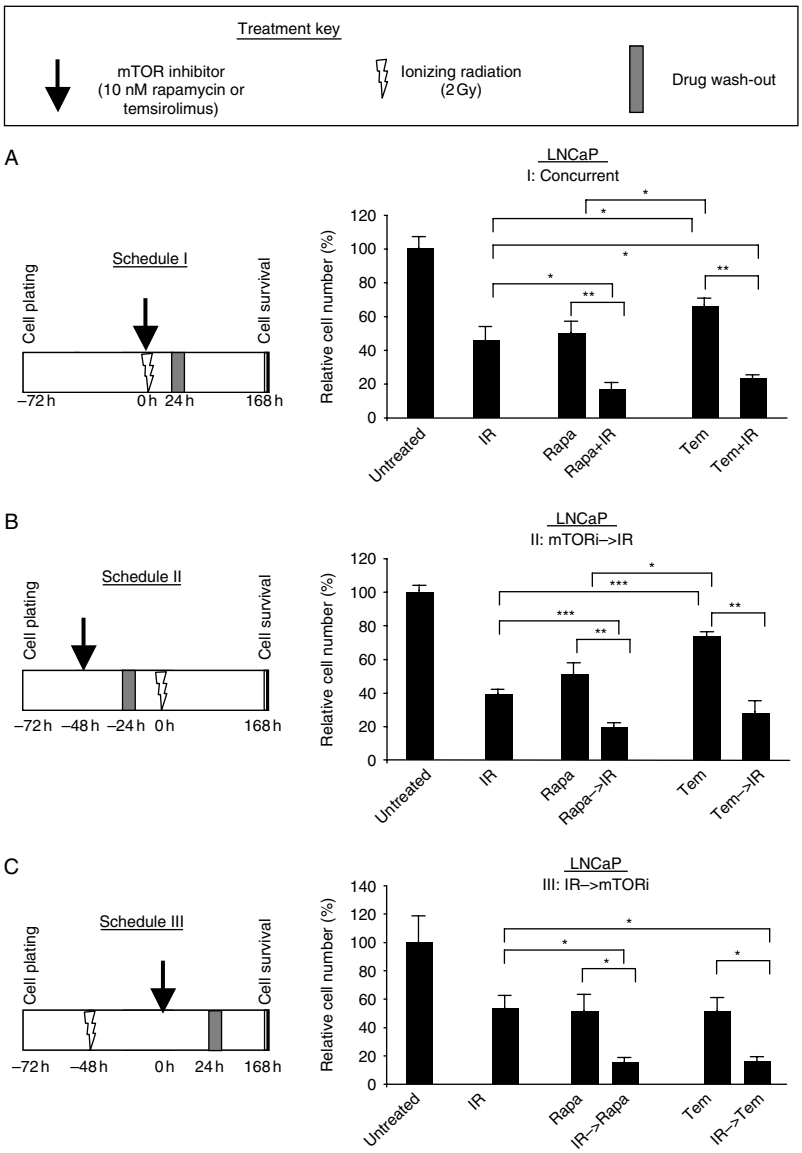

Figure 2 Schedule-specific radiosensitization of PCa cells by mTOR inhibition (A) Left panel: schematic of Schedule I treatment strategy (concurrent administration). As depicted, cells were seeded 72 h prior to final treatment, mTOR inhibitors, IR, or combination thereof were administered concurrently (Schedule I; set as time '0'), drug was washed out 24 h later, and cell number was assessed 168 h after treatment. Right panel: LNCaP cells were treated with 10 nM rapamycin (Rapa), 10 nM temsirolimus (Tem), 2 Gy IR (IR), combination of rapamycin and IR (Rapa + IR), combination of temsirolimus and IR (Tem + IR), or vehicle control (untreated). Cell survival in the untreated control was set to 100%; averages of three independent experiments and S.D. are shown. (B) Left panel: schematic of Schedule II (mTOR inhibitors as neoadjuvant). As depicted, cells were seeded 72 h prior to final treatment, administered 10 nM of either mTOR inhibitor, which was washed out of culture media 24 h later, then 24 h after wash, which at time 0 was exposure to 2 Gy IR, and cell number was assessed 168 h post-IR. Right panel: same as in (A), but with neoadjuvant mTOR inhibitor administration. (C) Left panel: schematic of Schedule III (mTOR inhibitors as an adjuvant post-IR). Cells were seeded 72 h prior to final treatment, administered 2 Gy IR, and then treated 48 h prior to final treatment (time 0), in this case it was either 10 nM rapamycin or temsirolimus. Right panel: same as in (A) and (B), but with adjuvant mTOR inhibitor administration. Statistical analysis of the indicated averages was performed using Student's *t*-test where $*P<0.05$, $**P<0.01$, and $***P<0.001$.

mTOR inhibition radiosensitizes CRPC cells

In addition to being used as a therapy for localized disease, IR is also used for local recurrence and metastases, when the cells have frequently become resistant to HT (CRPC cells). In the presence of androgens, mTOR inhibition sensitizes CRPC cells to IR (Fig. 3A). While there was less dependence on scheduling in this cellular context, Schedule III (adjuvant) remained the most effective in limiting cell doubling. To assess whether mTOR inhibition sensitizes CRPC cells to IR in a castrate environment, parallel studies were performed in steroid-depleted conditions. mTOR inhibitors retained the capacity to radiosensitize CRPC cells in the castrate condition (Fig. 3B), albeit to a lesser extent than observed in the presence of androgens (compared to Fig. 3A). Regardless, Schedule III remained the most effective, which suggests that there is a potential cell cycle component involved in the efficacy of the combination treatment. Since mTOR inhibitors alone can suppress AR-dependent cyclin D1 accumulation and cell cycle progression, it was hypothesized that these cytostatic effects underlie the scheduling effects of mTOR inhibitors.

Relative cell cycle inhibition in combination treatment is inversely correlated to efficacy of inhibiting population doubling

To examine the relative cell cycle distribution of cells in each of the treatment schedules prior to irradiation, the amount of DNA in cell populations was determined

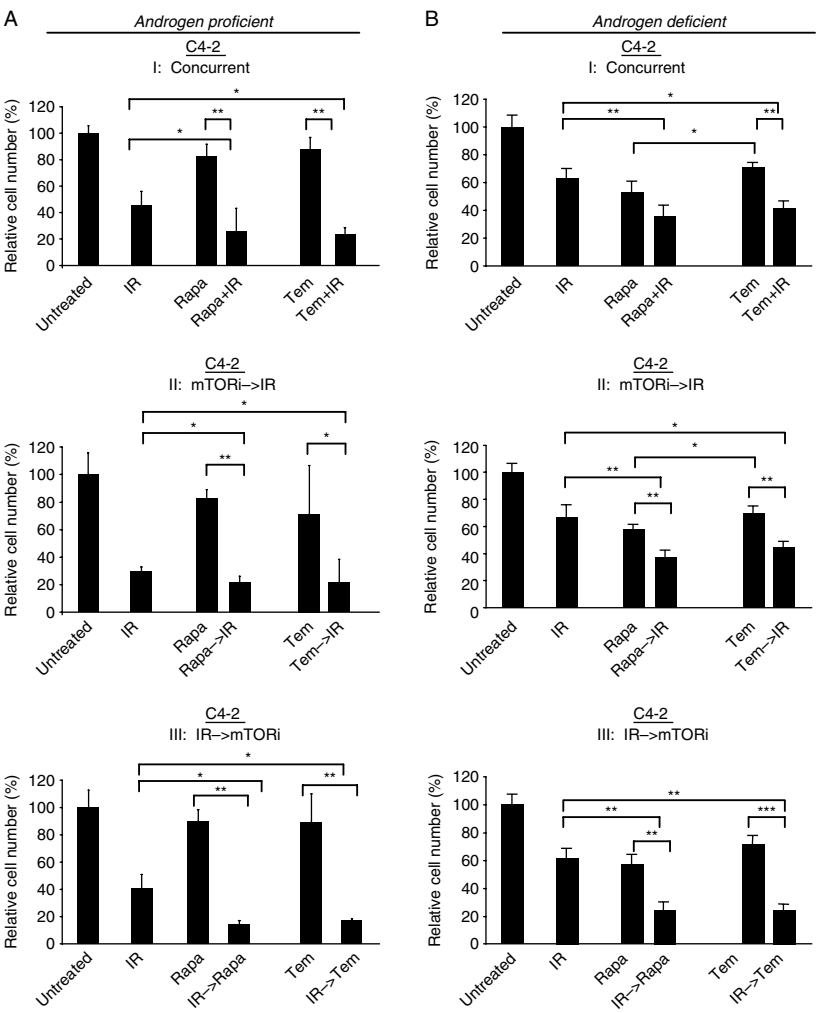

Figure 3 mTOR inhibitors sensitize CRPC cells to the effects of irradiation (A) C4-2 cells were treated as in Fig. 2. Top: mTOR inhibitor administration concurrent with IR (Schedule I). Middle: mTOR inhibitors administered as neoadjuvant to IR (Schedule II). Bottom: mTOR inhibitors administered as adjuvant to IR (Schedule III). (B) C4-2 cells were cultured in steroid-deprived media and treated as in (A). Cell survival in the untreated control is set to 100%, averages of three independent experiments and S.D. are shown. Statistical analysis of the indicated averages was performed using Student's *t*-test where $*P<0.05$, $**P<0.01$, and $***P<0.001$.

by flow cytometry. There was a significant increase in G_1 enrichment in both the concurrent and the neoadjuvant schedules compared with the adjuvant, with a concomitant decrease in G_2/M enrichment (Fig. 4A). This observed alteration in cell cycle distribution resulted in increased cells in a relatively radioresistant portion of the cell cycle (G_1; Yau *et al.* 1980) and a decrease in the number of cell in a relatively more radiosensitive portion of the cell cycle (G_2/M; Sinclair & Morton 1966) when mTOR inhibition was administered either concurrently or as a neoadjuvant. This same observation held true for CRPC cells as shown in Supplementary Figure 2A, see section on supplementary data given at the end of this article. In order to test the hypothesis that administration of mTOR inhibitors prior to the DNA-damaging insult of IR resulted in cytostatic effects that limited progression of cells to the radiosensitive cell cycle window, the relative change in S-phase progression was assessed for all treatment in the three schedules. There was significant inhibition of BrdU incorporation in both single-treatment mTOR inhibitor and IR in all schedules tested (Fig. 4A). The observed inhibition of S-phase progression was enhanced by combining mTOR inhibition and IR, but to a lesser extent in Schedule III (Fig. 4B). Representative PI/BrdU traces are provided in Supplementary Figure 2B, see section on supplementary data given at the end of this article. When these data were compared to the relative impact on cell number in Fig. 2 (Schedule III being the most effective in limiting population doubling), there was an apparent inverse correlation between relative cell cycle inhibition and inhibition of cell number. Therefore, while combinatorial treatment in Schedule III was least effective in limiting BrdU incorporation, it was this regimen that proved most effective in limiting cell number over time. To formally assess the impact of inhibition of cell cycle progression on relative treatment efficacy, cells were arrested with the DNA polymerase inhibitor aphidicolin (or not) prior to combination of mTOR inhibitors and IR (Schedule III). When the cell cycle was inhibited prior to administration of the Schedule III regimen, there was a significant alteration of the efficacy of combination

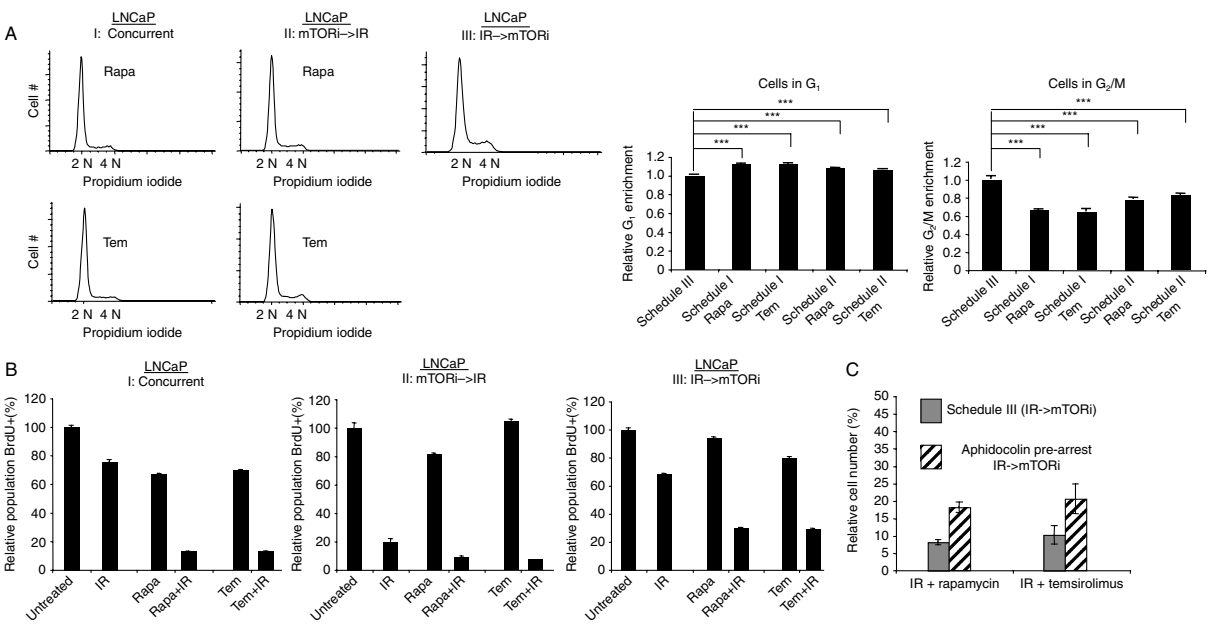

Figure 4 mTOR inhibitor-induced radiosensitization is a function of relative cell cycle inhibitory effect based on scheduling (A) Left panels: representative flow cytometry traces for each treatment Schedule that LNCaP cells were subjected to as depicted in Fig. 2 and harvested just prior to when IR would have been administered, fixed, and prepped for FACS analysis of DNA content as described in the Materials and methods section. Right panels: quantitation of the above. Data represented as relative G_1 (left) and G_2/M (right) enrichment averages and S.D. of at least three independent experiments. Schedule III (adjuvant) is set to 1. (B) LNCaP cells were treated according to the schemata depicted in Fig. 2, then 24 h post treatment, cells were harvested, fixed, and prepped for FACS analysis of BrdU incorporation and DNA content as described in the Materials and methods section. The data shown depicts the averages and S.D. of at least three independent experiments analyzing the percent of the cell population that is BrdU positive compared to untreated control, which is set to 100%. (C) LNCaP cells were either pre-arrested with vehicle control (gray bars) or aphidicolin (hashed bars) and then subjected to the mTOR inhibitors administered as an adjuvant to IR (Schedule III). Cell number was assessed 168 h after the last treatment by Trypan Blue exclusion and hemacytometer. Graph represents averages and S.D. of at least three independent experiments, with survival of untreated cells set to 100%. ***$P<0.001$.

treatment (Fig. 4C). Taken together, these data demonstrate that limiting cell cycle progression, either in the context of the scheduling or with another compound, prior to IR is less effective than using mTOR inhibitors in an adjuvant context. These data suggest that the anti-proliferative effect of mTOR inhibition prior to treatment is likely a detriment to therapeutic outcome, as the effects of IR may be greater in cells that are actively cycling, while the anti-survival effect of these compounds after IR may be of therapeutic benefit.

mTOR inhibition combined with IR hinders clonogenic PCa cell survival

To determine whether the observed mTOR inhibitor-mediated radiosensitization translated in long-term assays to significantly decrease in cell survival/clonogenicity, the Schedule III regimen was used in a clonogenic cell survival assay. Both Rapa and Tem when used in combination with IR significantly decrease clonogenic cell survival in HT-sensitive (Fig. 5A) and castration-resistant (Fig. 5B) cell models. These results, in a system that is a validated predictor of therapeutic response, indicate that adjuvant administration of mTOR inhibitors decreases PCa cell survival and replicative capacity.

Discussion

This study identifies mTOR inhibition as a therapeutic approach that, when combined with IR, suppresses cancer cell growth. While both IR and the two mTOR inhibitors tested (Rapa and Tem) showed single-agent efficacy in limiting PCa cell doubling at clinically relevant doses (Fig. 1), the data presented in this study provide evidence that when the combination of IR and mTOR inhibition is used, there is an additive effect in limiting both HT-sensitive PCa and CRPC cell doubling (Fig. 2). This cooperative effect was observed to be dependent on the scheduling of the treatment in that treatment of PCa cells with mTOR inhibitor(s) after IR treatment (adjuvant, Schedule III) resulted in the most additive effect as determined by both cell number and BrdU incorporation (Figs 2 and 4). Further, this observation was supported by the finding that arresting the cell cycle prior to administration of the most effective schedule reduced the efficacy of this treatment regimen (Fig. 4B). Finally, using clonogenic cell survival modeling, which is a predictor of *in vivo* efficacy (Wilson *et al*. 1984, Hirabayashi *et al*. 1987, Yung 1989), it was observed that adjuvant administration of either of the mTOR inhibitors tested resulted

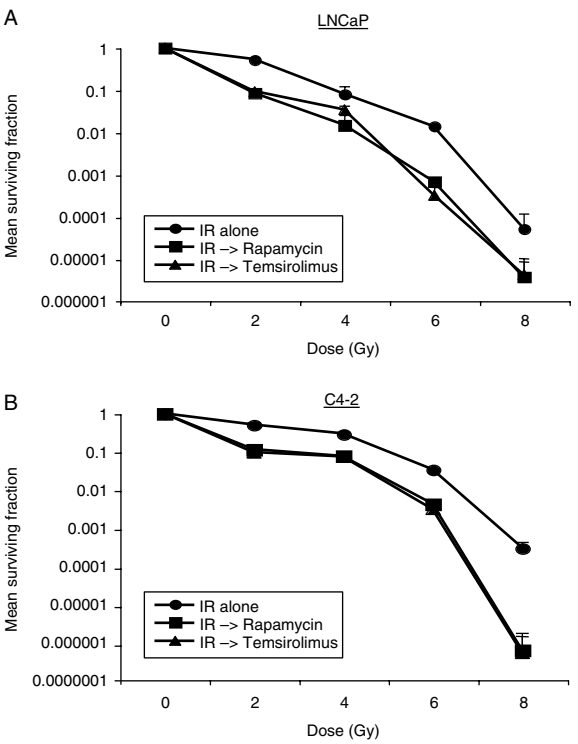

Figure 5 Clonogenic cell survival of both hormone therapy-sensitive and CRPC cells is reduced with mTOR inhibition following IR (A) LNCaP cells were serially diluted to appropriate concentrations and seeded in 50 ml tissue culture flasks. 24 h later, cells were exposed to indicated doses of IR. After 24 h, cells were treated with 10 nM rapamycin, 10 nM temsirolimus, or vehicle control. Cells were incubated for 14 days, fixed, stained with crystal violet, and colonies were counted. Colonies containing >50 cells were considered in the analysis. The data are represented on a semi-log scale, where the *x*-axis represents the dose of IR and the *y*-axis represents the mean surviving fraction and S.D. (B) C4-2 cells were seeded, treated, processed, counted, and analyzed as in (a).

in decreased replicative capacity of both HT-sensitive PCa and CRPC cells (Fig. 5). Only the schedule that proved to be most effective with respect to radiosensitization was utilized in the clonogenic assay. This was to ensure that any observed effects on sensitivity were not due to relative baseline plating efficiency to prior mTOR inhibitor administration. Together, these studies demonstrate that mTOR inhibition can radiosensitize PCa cells, and scheduling of the treatment alters the ultimate outcome as determined by both monitoring population doubling and clonogenic cell survival.

Despite the approved use of mTOR inhibitors for the treatment of renal clear cell carcinoma (RCC), there are few data regarding the impact of mTOR as a therapeutic target in PCa. However, a recent pharmacodynamic study (Armstrong *et al*. 2010) demonstrated that an mTOR inhibitor (Rapa) could be administered to men with localized PCa, attaining high intra-prostatic

levels of the compound with minimal adverse effects and effectively limiting mTOR signaling as determined by S6 kinase phosphorylation, which is a downstream effector of mTOR activity involved in protein translation. While there was little significant biological effect in these tumors with regard to cellular outcomes (as determined by the assessment of proliferative and apoptotic indices), this may have been a result of the short course of treatment (14 days). Nonetheless, these clinical data demonstrate the feasibility of targeting mTOR in PCa cells, thus revealing a potentially fruitful platform for combination therapy. There are currently a number of clinical trials at various stages, some using mTOR inhibitors as single agents and others in combination with agents such as docetaxel or AR-directed strategies (as reviewed in Morgan *et al.* (2009)); however, none of these trials are investigating the combinatorial use of IR and mTOR inhibition in PCa. While this study indicates some modest impact of mTOR inhibitors as single agents, the most significant anti-tumor activity was observed in combination with IR. Therefore, the data presented herein demonstrating the radiosensitization of both HT-sensitive and CRPC cells in the clonogenic cell survival assay emphasize the importance of considering treatment schedule and provide the basis for clinical investigations. These data present a substantive advance, as there are no clinical agents currently approved, which confer sensitization to RT aside from androgen deprivation therapy (ADT). Of note, in CRPC cells cultured in conditions mimicking ADT, mTOR inhibition served as a radiosensitizer as well.

A critical finding herein was that the efficacy of mTOR inhibitors as a means to radiosensitize was significantly influenced by treatment schedule in both HT-sensitive PCa and CRPC cells. The evidence shown suggests that the observed schedule dependence can be attributed to the impact of mTOR inhibitors on cell cycle progression. The G_1 arrest induced by mTOR inhibitors prior to IR protected against radiation-induced cellular outcomes, whereas mTOR inhibitors in the adjuvant setting resulted in a more robust decrease in cell doubling. Interestingly, the effect of mTOR inhibitors was not influenced by PTEN status, as both PTEN-proficient (LAPC4) and PTEN-deficient (LNCaP) cells exhibited similar response to schedule-dependent combination therapy. Moreover, the impact of mTOR inhibition on radiosensitization was independent of p53 status as, in contrast to LNCaP cells, the LAPC4 model system lacks functional p53 (van Bokhoven *et al.* 2003). Schedule-dependent sensitization to DNA damaging therapies by mTOR inhibition is not without precedent. It has been demonstrated that co-treatment of doxorubicin with an mTOR inhibitor was synergistic in T-cell lymphoma *in vitro* (Huang *et al.* 2010), as was adjuvant administration of mTOR inhibitor, compared to neo-adjuvant mTOR inhibition with these agents, which resulted in no synergistic effect on cellular outcomes. Additionally, it has been demonstrated that Tem administered to HT-sensitive PCa cells after docetaxel was more effective in limiting clonogenic cell survival, compared with concomitant treatment (Fung *et al.* 2009). These collective observations underscore the importance of assessing the impact of sequencing when combining mTOR inhibitors with genotoxic agents, especially with regard to the relative impact of these agents to alter cell cycle inhibition. As demonstrated herein, administration of mTOR inhibitors prior to radiation results in larger proportions of the cell populations being in relatively radioresistant portions of the cell cycle (G_1) and fewer cells in radiosensitive portions (S and G_2/M).

As demonstrated herein, clinically relevant doses of both Rapa and Tem exhibit single-agent cytostatic and cytotoxic effects in PCa cells and conferred schedule-dependent radiosensitization. The underlying mechanism(s) by which adjuvant administration of mTOR inhibition sensitizes cells to RT is the focus of ongoing investigation. Recently, it was demonstrated that mTOR is directly involved in the repair of DNA damage with respect to double-strand breaks, which occur frequently in cells exposed to IR (Chen *et al.* 2010), and these effects could therefore contribute to the radiosensitization observed in this study. Consonantly, it has been demonstrated that mTOR inhibition confers radiosensitization phenotypes in multiple tumor types (Ekshyyan *et al.* 2009, Nagata *et al.* 2010, Saunders *et al.* 2010), and that mTOR inhibition radiosensitizes soft tissue sarcoma and tumor vasculature (Murphy *et al.* 2009), which could have a similar impact on the response to RT. mTOR inhibitors also show cooperative effects with RT-independent DNA damaging agents, including doxorubicin (in T-cell lymphoma (Huang *et al.* 2010)), 5-fluorouracil and/or docetaxel (in gastric cancer (Matsuzaki *et al.* 2009)), carboplatin and paclitaxel (in head and neck cancer (Aissat *et al.* 2008)), and cisplatin (in hepatocellular carcinoma (Aissat *et al.* 2008)). In PCa cells, limited evidence suggests that mTOR inhibition can confer sensitization to doxorubicin (Grunwald *et al.* 2002), and combining mTOR inhibitors with docetaxel has been shown to be effective in limiting PCa cell growth *in vitro* and *in vivo* in a schedule-dependent manner (Fung *et al.* 2009). While mTOR inhibitors have been shown to

cooperate with DNA damage in AR-negative PCa cells both *in vitro* and *in vivo* (Wu *et al*. 2005, Cao *et al*. 2006), the relevance of these models to the majority of human tumors, which retain AR, remains uncertain. One study has demonstrated that mTOR inhibition and docetaxel administration is an effective combination in an intra-tibial AR-positive model of PCa (Morgan *et al*. 2008), while the other has shown that combining mTOR inhibition and AR antagonistic therapy results in PCa cell apoptosis and delayed progression to castration resistance (Schayowitz *et al*. 2010). As such, mTOR inhibitors appear to harbor the capacity to improve responses to RT and selected DNA damage-inducing therapeutics, as well as AR-directed strategies.

In summary, the studies presented herein demonstrate that mTOR inhibitors exhibit schedule-dependent effects on the RT response in PCa cells and confer significant radiosensitization effects when used in the adjuvant setting. Remarkably, the effects of mTOR inhibition as a means to achieve radiosensitization was conserved in both the HT-sensitive PCa and the CRPC settings, thus indicating that mTOR inhibitors may be an effective means to improve response to DNA damage-inducing therapeutic regimens in advanced disease. Combining these data herein provide the foundation for clinical investigation and illuminate new means by which PCa treatment may be improved.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0072.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M J S, M A A, Y R L, A P D, and K E K conceived and designed the experiments. M J S, R D, D T H, and M A A performed the experiments. M J S, R D, D T H, Y R L, A P D, and K E K analyzed the data. K E K contributed reagents or analysis tools. M J S and K E K wrote the paper.

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Appendix B

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ORIGINAL ARTICLE

Targeting cell cycle and hormone receptor pathways in cancer

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The cyclin/cyclin-dependent kinase (CDK)/retinoblastoma (RB)-axis is a critical modulator of cell cycle entry and is aberrant in many human cancers. New nodes of therapeutic intervention are needed that can delay or combat the onset of malignancies. The antitumor properties and mechanistic functions of PD-0332991 (PD; a potent and selective CDK4/6 inhibitor) were investigated using human prostate cancer (PCa) models and primary tumors. PD significantly impaired the capacity of PCa cells to proliferate by promoting a robust G_1-arrest. Accordingly, key regulators of the G_1-S cell cycle transition were modulated including G1 cyclins D, E and A. Subsequent investigation demonstrated the ability of PD to function in the presence of existing hormone-based regimens and to cooperate with ionizing radiation to further suppress cellular growth. Importantly, it was determined that PD is a critical mediator of PD action. The anti-proliferative impact of CDK4/6 inhibition was revealed through reduced proliferation and delayed growth using PCa cell xenografts. Finally, first-in-field effects of PD on proliferation were observed in primary human prostatectomy tumor tissue explants. This study shows that selective CDK4/6 inhibition, using PD either as a single-agent or in combination, hinders key proliferative pathways necessary for disease progression and that RB status is a critical prognostic determinant for therapeutic efficacy. Combined, these pre-clinical findings identify selective targeting of CDK4/6 as a *bona fide* therapeutic target in both early stage and advanced PCa and underscore the benefit of personalized medicine to enhance treatment response.

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INTRODUCTION

Prostate cancer (PCa) is a leading cause of cancer mortality in men and presents an ongoing therapeutic challenge.[1] PCa is one of the most prevalent cancers diagnosed in men with a lifetime risk of 1-in-6 according to the American Cancer Society. Localized disease can be effectively managed through surgery and/or radiation;[1,2] however, advanced disease represents a major clinical challenge as standard chemotherapeutics are not typically effective.[1] Treatment for advanced PCa is based primarily on its dependence of the androgen receptor (AR) for development, growth and survival. Therefore, the mainstay of treatment for advanced disease involves androgen deprivation therapies (ADTs) that result in cell cycle arrest and/or death.[1] These ablative therapies are effective on average for 2–3 years, whereupon lethal castration-resistant PCa (CRPC) develops, because of re-activation of AR signaling.[1] Despite recent progress, the ability to effectively treat CRPC remains limited; therefore, additional therapeutic options are needed.

Owing to the importance of androgen signaling, PCa at all stages remains reliant on AR to promote growth and/or survival.[1] AR is a nuclear hormone receptor and upon activation by androgens (for example, dihydrotestosterone, (DHT)) induces a complex transcriptional program that includes proliferation and expression of prostate-specific genes such as *kallikrein-related peptidase 3* (*KLK3*) (prostate-specific antigen, PSA). As an AR-target, *KLK3*/PSA is not only used as a surrogate for AR activity, but is also used in the clinical setting to monitor disease.[1] Treatment of advanced PCa entails limiting AR activity either through inhibiting androgen levels or through the use of direct AR antagonists like bicalutamide.[1] Recurrent disease invariably ensues as a result of re-activated AR, indicated by resurgent *KLK3*/PSA. Multiple mechanisms have been described to explain restored AR activity including: amplification, activating mutations or splice variants, altered post-translational modifications, aberrant expression of cofactors, and intracrine androgen synthesis.[1] Regardless of the mechanism of restoration, AR continues to promote proliferation in advanced disease. Thus, a concerted effort has been undertaken to determine how AR governs cell cycle progression in order to target the cell cycle machinery and improve therapy.

The mechanism by which androgen/AR induces the cell cycle to instigate proliferation has been recently reviewed.[3] Briefly, androgen promotes enhanced translation, through mammalian target of rapamycin, of the D-type cyclins and the induction of $p21^{Cip1}$ mRNA. These inductive events combine to assist the formation of an active complex between D-cyclins, $p21^{Cip1}$ and the cyclin-dependent kinases 4/6 (CDK4/6) that are important for cell cycle progression. The combined kinase functions of early G_1 cyclin-D/CDK4 or 6 and late G_1 cyclin-E/CDK2 serve to phosphorylate the retinoblastoma protein (RB), allowing E2F transcription factors to control downstream cyclin expression (for example, cyclin A) required for S-phase transition. Given the importance of the cyclin/CDK/RB-axis in controlling the G_1-S transition in the majority of cancers, including PCa, a prime

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therapeutic candidate has been CDK activity.[4–7] In PCa, the majority of early pre-clinical studies designed to modulate CDK activity with flavopiridol, a pan-CDK inhibitor, showed antitumor activity in xenografts.[8–10] However, enthusiasm waned as phase II clinical trials of flavopiridol were disappointing, due largely to off-target effects and toxicity.[11] Recently, a clinical grade, orally active CDK inhibitor (PD-0332991, PD) has been developed that selectively and reversibly inhibits CDK4/6 at low nanomolar concentrations.[12,13] Pre-clinical studies in other cancer models have shown that PD induces a robust cytostatic G_1-arrest, delays or prevents xenograft formation with minimal cell death, and may hinder metastatic potential. PD has been shown preliminarily to limit disease progression in patients with inoperable RB-positive teratomas, which are commonly resistant to chemo- and radiation therapy, with minimal adverse events.[14] Importantly, clinical trials have been initiated based on pre-clinical data from both hematological and solid tumors (for example, lymphoma,[15] leukemia,[16,17] myeloma[18,19], breast,[20–25] colon,[26–28] lung,[29] esophageal[30] and glioblastoma[31,32]).

Currently, no study has assessed the efficacy of PD in PCa, despite the preliminary pre-clinical analyses and few recently completed phase I trials.[33–35] Here, a pre-clinical evaluation of PD was undertaken to determine its therapeutic potential in PCa. Using well-established hormone-dependent and CRPC cell models, PD showed remarkable single-agent activity with regard to limiting cellular proliferation and growth. Subsequent analyses demonstrated the feasibility of combinatorial therapy between PD and existing treatments such as AR antagonists and radiation. The potential therapeutic effect of PD was revealed using both *in vivo* mouse xenografts and a recently developed novel *ex vivo* assay using primary human tumors obtained by radical prostatectomy. These pre-clinical findings, using PD, suggest selective CDK4/6 inhibition as a potential node of intervention in PCa, and warrant future studies to evaluate its clinical efficacy.

RESULTS

PCa cell proliferation is attenuated by CDK4/6-specific inhibition

PD, a CDK 4/6-selective inhibitor, was evaluated in a comprehensive panel of hormone-sensitive PCa cells. Dose dependence studies for PD indicated an IC_{50} range of 44–91 nM (Supplementary Figure 1A) consistent with other hormone-dependent cancer cell systems.[20,36,37] PCa cells were treated with PD ($\sim$5–10X the IC_{50}) and assessed for active proliferation via pulse labeling with bromodeoxyuridine (BrdU) and quantified by flow cytometry (Figure 1a). As shown, BrdU incorporation in LNCaP, LAPC4 and VCaP cells was profoundly attenuated (treated vs control (%): 4.27 vs 23.1, 2.93 vs 28.5 and 2.32 vs 23.2, respectively). Cell cycle analyses revealed a strong G_0/G_1-phase arrest (data not shown) consistent with suppression of CDK4/6 activity.[5] VCaP cells treated with PD, which showed the strongest anti-proliferative response, displayed minimal cell death as indicated by sub-G1 accumulation (Supplementary Figure 1B) and cleaved poly ADP-ribose polymerase (PARP) (Supplementary Figure 1C) as compared with etoposide. Similarly, PD had minimal impact on extracellular signal-regulated kinase signaling (Supplementary Figure 1D). In addition, treatment of PD conferred a reduction in cell growth as indicated by crystal violet staining (Figure 1b). As the cyclin/CDK/RB pathway is implicated in oncogenic signaling in cancer,[38] protein expression of cell cycle components was monitored after PD treatment (Figure 1c). In all cells tested, protein levels of CDK4 and AR were unchanged by PD. In contrast, RB protein Ser780-phosphorylation, a known site of CDK4/6 activity,[38] was suppressed. Cyclin A, a well-characterized RB target gene and positive indicator of proliferation,[38,39] levels were attenuated by PD. Combined, the decreased RB phosphorylation and cyclin A protein levels strongly indicated that PD effectively inhibited CDK4/6 activity. Examination of the protein levels of key G_1-cyclins (cyclins D1 and E), required for the activation of CDKs (CDK4/6 and CDK2, respectively), revealed disparate and cell-specific changes on PD exposure. Cyclin E1 was unchanged or decreased only in LAPC4 cells, whereas cyclin D1 was modestly but significantly increased in LNCaP and LAPC4 but not VCaP cells. Elevated cyclin D1 was somewhat surprising, as many therapeutics that suppress proliferation and induce G_1-arrest are frequently associated with loss of cyclin D1.[40] As cyclin D1 binds and initiates CDK4/6 activity,[38,41,42] co-immunoprecipitation analyses were performed (Supplementary Figure 1E) to determine if PD altered the cyclin D1–CDK4 complex. Immunoprecipitation of CDK4 from PD-treated LNCaP cells resulted in a modest increase in co-immunoprecipitated cyclin D1 (compare lanes 2 and 5), suggesting that PD may stabilize an inactive cyclin D1–CDK4 complex and hinder the turnover of cyclin D1. Combined, these data indicate that PD inhibits CDK4/6-dependent phosphorylation of RB resulting in suppression of proliferation/growth in multiple hormone-sensitive PCa cells.

Efficacy of AR-directed therapeutics is retained in combination with CDK4/6 inhibition

Virtually all stages of PCa are dependent on androgen/AR signaling.[1] Consequently, advanced PCa is treated with hormone-based therapies that block AR signaling.[1] It has been shown that aberrant cyclin D1 levels can selectively modulate androgen-dependent AR activity.[43] Therefore, the impact of PD on androgen-dependent AR activity and/or potential response to AR-directed therapies (i.e., casodex, Csdx) was assessed via gene expression analyses of AR-target genes (*KLK3*/PSA, *TMPRSS2* and *KLK2*) with known clinical relevance (Figure 2). To measure androgen/AR-dependent target gene expression, LNCaP (Figure 2a), LAPC4 (Figure 2b) and VCaP (Figure 2c) cells were cultured in steroid hormone-depleted (charcoal dextran-treated serum) media, then stimulated with DHT in the presence of the AR antagonist Csdx, PD or a combination thereof. As expected, DHT resulted in a robust increase in the mRNA expression of all AR-target genes. Conversely, Csdx significantly reduced DHT-induced AR-target gene expression. Addition of PD (or in combination with Csdx) had no impact on DHT-induced gene expression in VCaP cells and had minimal cooperative impact in LNCaP and LAPC4 cells, consistent with the established observation that cyclin D1 modulates AR activity independent of CDK function. These data indicate that PD acts in a manner distinct from AR-directed therapeutics, and that suppression of CDK4/6 activity does not antagonize standard of care AR-directed therapies.

CDK4/6-specific inhibition sensitizes PCa cells to ionizing radiation (IR)

Radiation therapy in conjunction with novel therapeutics is frequently used to treat locally advanced PCa.[1,2,44] Therefore, the capacity of PD to cooperate with IR was assessed (Figure 3). As expected, single-agent treatment with PD or IR (compared with vehicle) suppressed long-term cell growth (at day 7) indicated by *in vitro* cell growth kinetics of LNCaP, LAPC4 and VCaP cells (Figure 3a) and parallel colony formation assays (Supplementary Figure 2). Concurrent treatment with PD and IR resulted in a significant attenuation in cell growth (compared with either single-agent alone) for LNCaP, LAPC4 and VCaP cells. All cell lines (Table 1, data calculated from the raw data in Figure 3a) demonstrated prolonged doubling times (compared with vehicle) either on individual (PD: 1·2- to 1·6-fold or IR: 1·4- to 2.0-fold) or combined treatments (PD + IR: 2.4- to 3.7-fold). PD treatment alone substantiated the above findings that CDK4/6-specific inhibition suppresses proliferation. In addition, the ability of PD to cooperate with IR was determined using a clonogenic survival assay (Figure 3b). Importantly, these studies further demonstrated

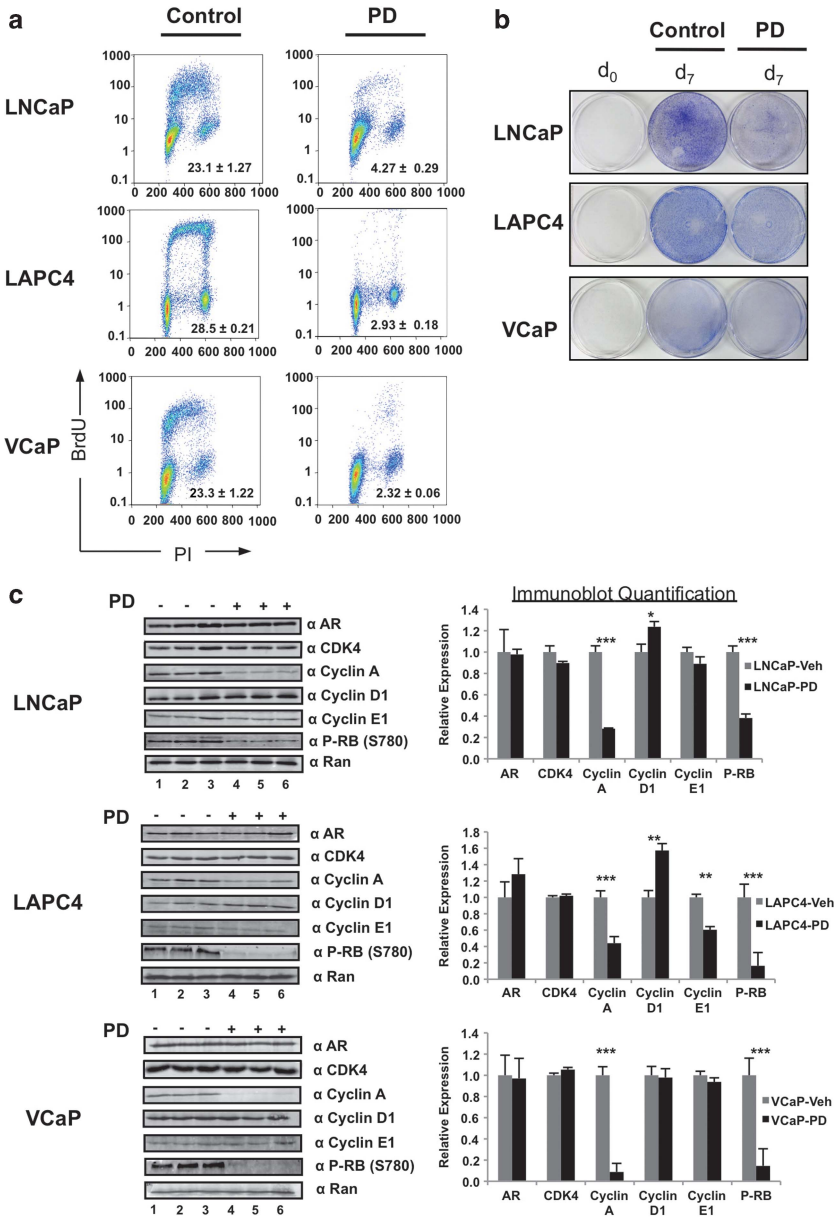

Figure 1. CDK4/6-specific inhibition suppresses proliferation of androgen-dependent PCa cells. The impact of the CDK4/6-specific inhibitor (PD) on proliferation and cell cycle components was characterized in multiple androgen-dependent PCa cell model systems. (**a**) Bivariate flow cytometry analyses of: LNCaP (upper), LAPC4 (middle) and VCaP (lower) cells treated 24 h with 0.1% dimethylsulphoxide control (left column) or 0.5 μM PD (right column). Profiles are representative of three independent experiments. The x axis denotes relative DNA content as indicated by propidium iodide (PI) staining. The y axis denotes cells undergoing active S-phase as indicated by 2-h pulse-label of BrdU. Inset values: % BrdU incorporation (mean ± s.d., from an experiment performed in biological triplicate). (**b**) Crystal violet staining at day 7 (d_7) relative to plating at day 0 (d_0) from LNCaP, LAPC4, and VCaP cells initially treated with control or 0.5 μM PD for 24 h. Data are representative of three independent experiments. (**c**) Immunoblot analyses, from parallel treated cells in panel (**a**) for the indicated prostate and cell cycle components (left panels) and quantified by LI-COR image analyses (right panels). Loading and quantification are relative to Ran. Grey and black bars = control and PD treatments, respectively. *; **; *** indicates *P*-values: <0.05; 0.01; 0.001, respectively.

that the combination of PD and IR (at 2 or 4 Gy, doses higher than 6 Gy yielded no colonies (data not shown)) significantly reduced the number of colonies formed as compared with IR treatment alone. Combined, these data indicate that PD cooperates with and/or sensitizes PCa cells to the effects of IR.

CRPC cells are amenable to CDK4/6-specific inhibition, dependent on RB status

Based on the findings above that PD showed a remarkable capacity to not only inhibit proliferation but also act in concert with IR to limit growth of hormone-sensitive PCa cells, it was surmised that CDK4/6-specific inhibition could be advantageous in the treatment of CRPC, which is typically associated with increased proliferative/survival capacity and poor outcome.[1] To challenge this hypothesis, a collection of diverse CRPC cells were treated with PD, and flow cytometry and gene expression analyses were performed to evaluate the overall response of CRPC cells to therapy (Figure 4). Cell cycle analyses of PC3M cells (Figure 4a), a variant of the PCa-derived PC3 cell line with bone homing potential, indicated that PD treatment resulted in an increased percentage of cells in G_0/G_1 with a concomitant decrease in S and

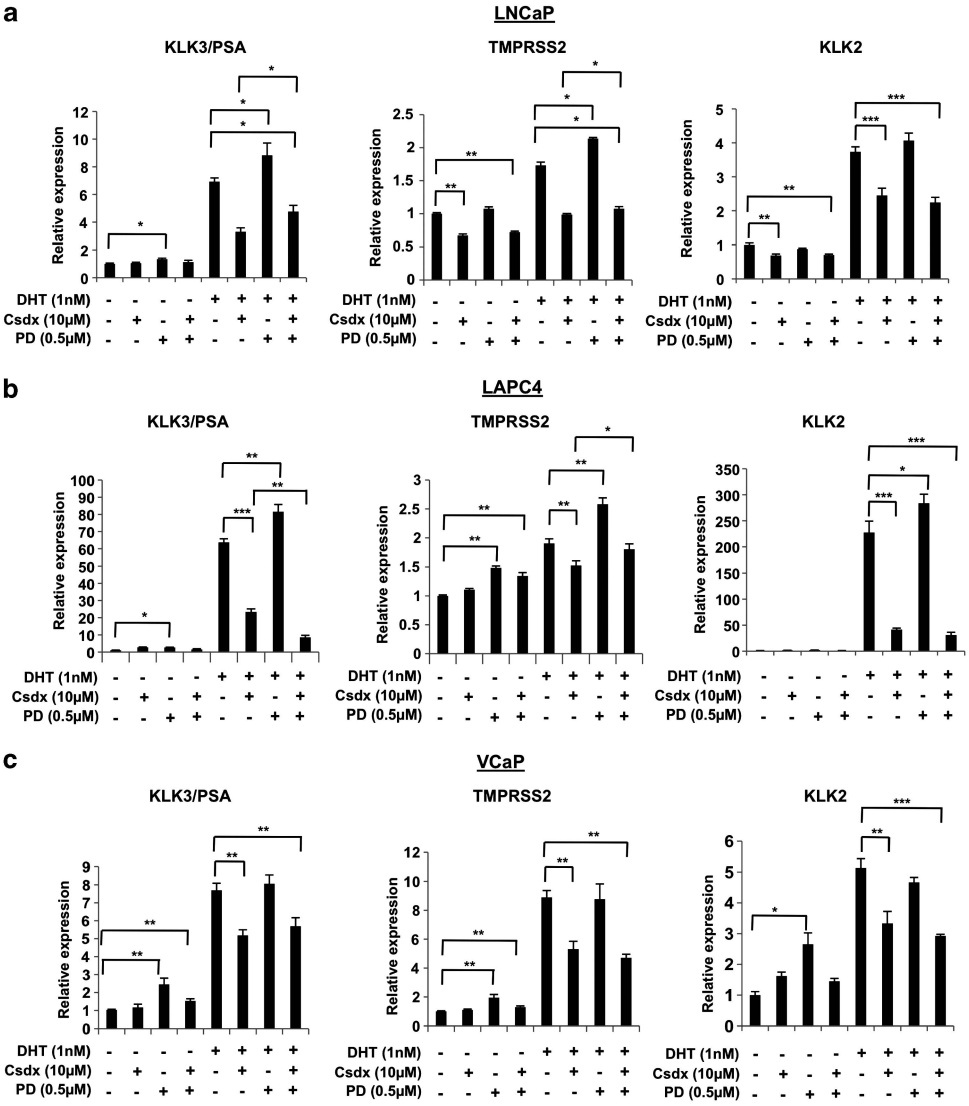

Figure 2. AR-directed therapies are effective in the presence of CDK4/6-specific inhibition. To assess AR activity, androgen-dependent PCa cells: (**a**) LNCaP (**b**) LAPC4 and (**c**) VCaP were cultured 72 h in media containing steroid-deprived serum (5% charcoal-dextran treated (CDT)) then stimulated 24 h with (or without) DHT (1 nM) in the presence of PD (0.5 μM), Csdx (10 μM) or combination of PD and Csdx. Relative mRNA expression normalized to glyceraldehyde 3-phosphate dehydrogenase was determined by quantitative PCR (qPCR) for the known AR-target genes: *KLK3*/PSA (left), *TMPRSS2* (middle) and *KLK2* (right). Indicated treatments for each gene are relative to non-DHT and non-drug treated cells. *; **; *** indicates *P*-values: <0.05; 0.01; 0.001, respectively.

G_2M (treated vs control (%): 86.1 vs 61.7, 8.27 vs 28.8, 3.24 vs 8, respectively). PC3M cells, because of a lack of detectable AR, represent a rare form of CRPC.[45] These data not only demonstrate the effectiveness of PD in multiple forms of PCa, but also are consistent with the above findings (Figure 2) that the effects of PD on proliferation are independent of AR.

Although PD was effective at limiting AR-negative CRPC cell proliferation, the vast majority of CRPC cases remain AR positive.[1] Therefore, it is essential to evaluate PD in the context of CRPC cells that maintain AR. Based on the mechanism of action of PD, it was hypothesized that the capacity of PD to limit CDK4/6-dependent proliferation is dictated by the status of RB. Recently, isogenic PCa tumor models of RB loss were developed, wherein it was shown that RB loss is a critical mediator of the transition to castration resistance, and promotes lethal phenotypes through enhanced AR levels and activity.[46] Thus, using isogenic pairs derived from LNCaP cells with and without RB knockdown, the notion that RB is required for PD action was assessed. Importantly, cell cycle analysis of this CRPC model system treated with PD (Figure 4b) indicated that RB loss is sufficient to promote PD resistance. Moreover, the ability of RB loss to promote therapeutic bypass of PD was confirmed by stable transduction of the shRB1 construct into PC3 (which lack AR), LAPC4 and VCaP cell model systems (Supplementary Figure 3). Thus, in these diverse cellular contexts, the data overwhelmingly support the concept that the effectiveness of PD is dependent on the integrity of RB.

Additional studies were performed using two common CRPC cells (22Rv1 and LNCaP-derived C4-2, both positive for RB and AR) that represent different pathways of acquired resistance to hormone-based therapy. For these studies, 22Rv1 and C4-2 cells (Figure 4; panels c and d, respectively) were cultured and treated in conditions that mimic ADT. As expected, both cell models proliferated with control treatments as indicated by the percentage of cells in S-phase. Treatment of both 22Rv1 and C4-2 cells with PD resulted in an increased percentage of cells in G_0/G_1 (treated vs control (%)—22Rv1: 85.6 vs 55, C4-2: 85.1 vs 69.2)

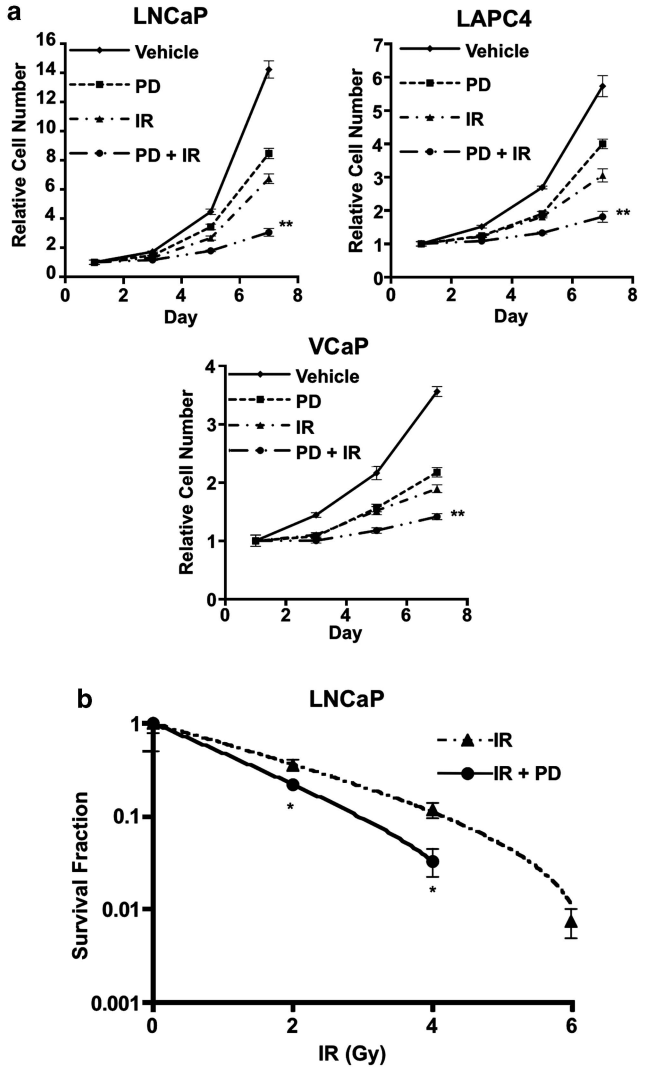

Figure 3. CDK4/6-specific inhibition cooperates with IR to attenuate PCa cell growth. IR was administered in conjunction with PD to evaluate the effect of combinatorial therapy, as described in the Material and methods section. (**a**) Cell growth analyses of LNCaP, LAPC4 and VCaP cells treated with single-dose IR (2 Gy), PD (0.5 μM) or a combination of both. Cell number, for the indicated times and treatments, was determined by Trypan blue exclusion and was normalized to the initial day of treatment (day 1). **Indicates a *P*-value <0.01 relative to individual treatments alone. Data shown are representative of three independent experiments. (**b**) Clonogenic assay using LNCaP cells treated with the indicated dose (Gy) of IR or in combination with PD (0.5 μM). Colonies were stained with crystal violet and counted 14 days post-treatment. Studies were performed in biological triplicate and are representative of three independent experiments. *Indicates a *P*-value <0.05 relative to IR treatment alone.

Table 1. Doubling times

	LNCaP	*LAPC4*	*VCaP*
Veh	1.57[a]	2.38	3.28
PD	1.95	3.00	5.36
IR	2.18	3.73	6.62
PD + IR	3.72	7.00	12.0

Abbreviations: IR, ionizing radiation; PD, PD-0332991. [a]Values in days.

paralleled by decreased S (treated vs control (%)—22Rv1: 10.2 vs 34.1, C4-2: 6.77 vs 18) and G_2M (treated vs control (%)—22Rv1: 4.27 vs 10.9, C4-2: 6.21 vs 8.47). To evaluate the impact of PD on AR signaling and potential utility in combination with ADT in CRPC disease, gene expression analyses were performed using C4-2 cells (Figure 4e). AR-target (that is, *KLK3*/PSA and *TMPRSS2*) gene expression was similar to hormone-sensitive LNCaP cells (Figure 2), suggesting that PD does not interfere with ADT in the CRPC setting. These data demonstrate that PD effectively attenuated proliferation of multiple CRPC models, independent of AR status and/or standard ADT therapy. Moreover, PD action is dependent on RB, suggesting the need to stratify patients based on RB status.

CDK4/6-specific inhibition impacts PCa tumor proliferation both *in vivo* and in *ex vivo* primary human tumors

Although CDK4/6-specific inhibition is effective against androgen-dependent and CRPC cell proliferation or growth *in vitro*, additional analyses were performed to discern the *in vivo* benefit of PD treatment in PCa systems (Figure 5). For these studies, VCaP cells were used as they harbor two clinically relevant aberrations: (1) elevated AR that is frequently associated with disease progression, and (2) chromosomal rearrangements that fuse ETS oncogenes (*ERG* or *ETV*) under androgen/AR control via the *TMPRSS2* regulatory locus as seen in 50–75% of PCa.[47] Mice harboring VCaP xenografts were treated with PD (150 mg/kg) or lactate vehicle, consistent with known dosage regimens.[18,22,25,28,48] VCaP tumor proliferation was determined using immunohistochemistry against endogenous Ki-67 (Figure 5a, left panel). As expected, VCaP tumors in control treated animals were proliferative. In contrast, treatment with PD resulted in a reduction in Ki-67. Quantification (Figure 5a, right panel) revealed a significant reduction (65.8%) in VCaP proliferation upon PD treatment. As the data in Figure 4 indicated that RB is a critical factor for PD action, *in vivo* xenograft growth was evaluated using the PC3 cells (described in Supplementary Figure 3) treated with a short course of PD. As shown in Supplementary Figure 4A, PC3-shNS cells had a delayed growth response following treatment with PD. These data were expected based on previous studies in other model systems.[13,22,24,25,32] In contrast, PC3-shRB1 cells showed a growth profile similar to that of vehicle-treated PC3-shNS cells. Together, these *in vivo* data indicate that PD is sufficient to reduce xenograft proliferation and delay growth in an RB-dependent manner.

Encouraged by the xenograft data, additional studies were performed using a novel *ex vivo* culture system.[49] Human prostatectomy tissues (processed into $\sim 1\,mm^3$ explants) were cultured, using standard cell culture growth media and components, on sterile dental sponges to allow for efficient media/oxygen exchange. Using these conditions the tumor tissue maintains many of its characteristics including: histology, AR status, proliferative capacity and stromal environment.[49] This *ex vivo* assay affords the potential to assess novel therapies in tissue that is one-step removed from the patient without the difficulty and expense of implanting tissue into animals. In proof of concept, we obtained tissue specimens from five patients that were subsequently determined to be RB proficient (Figure 5b, left panel; representative RB-positive tumor and corresponding hematoxylin and eosin). Explant tissues were treated, as described in the Materials and methods section, for a short- and long-term duration (days 2 and 6, respectively) in the presence of two different concentrations of PD (0.5 and 1.0 μM). As shown (Figure 5b, right panel; same specimen as left panel), *ex vivo* culture for 6 days in control or two concentrations of PD did not overtly disrupt the glandular structure as indicated by hematoxylin and eosin staining. Similarly, minimal cell death was observed as determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, in both long-term control and treated

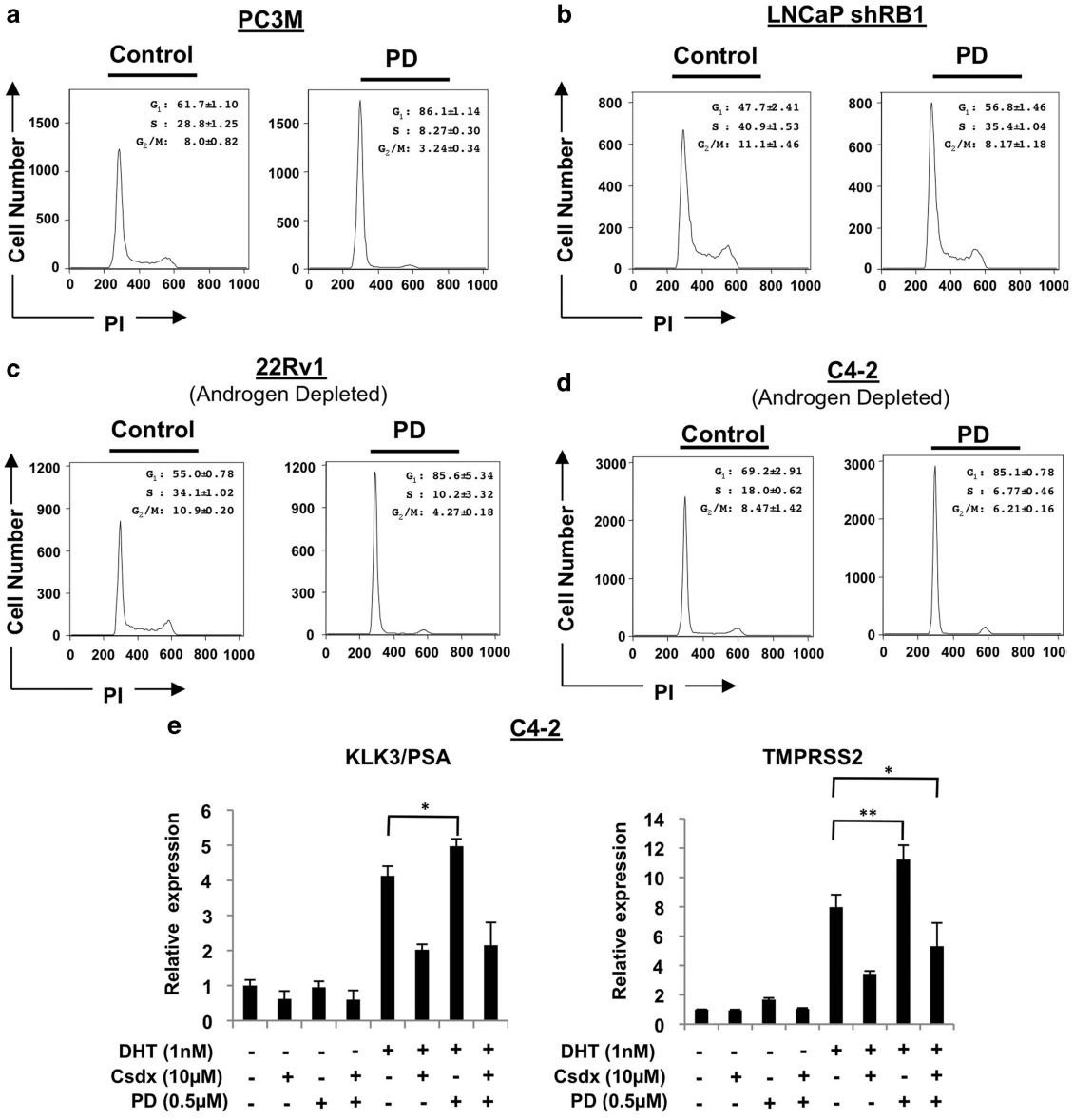

Figure 4. CRPC cells are sensitive to CDK4/6-specific inhibition, dependent on RB status. The relevance of PD treatment to advanced PCa was determined using multiple CRPC cell model systems. Flow cytometry was performed on CRPC cells: (**a**) PC3M; (**b**) LNCaP shRB1 (stable LNCaP cells depleted of the RB protein); (**c**) 22Rv1; and (**d**) C4-2 treated with control (left panels) or PD (right panels), as described in Figure 1a. Representative flow cytometry profiles for each cell model system are shown. The y axis denotes the number of fixed cells stained with propidium iodide (PI). Inset values: % PI-stained cells (mean ± s.d., from an experiment performed in biological triplicate) in the G_1-, S-, G_2M-phases as determined using the cell cycle algorithm in FlowJo. Note: 22Rv1 and C4-2 cells were treated in media containing 5% charcoal-dextran treated (CDT) serum, as described in the Materials and methods section. (**e**) Gene expression analyses from C4-2 cells was performed, as described in Figure 2, to assess the impact of indicated therapies on AR activity. Representative AR target genes: *KLK3*/PSA (left) and *TMPRSS2* (right) are shown. *; ** indicates *P*-values: <0.05; 0.01, respectively.

explants (data not shown). Ki-67 staining, described in the Materials and methods section, indicated that both PD concentrations reduced proliferation compared with the control. As shown (Figure 5c), short-term treatment with 1 μM PD significantly inhibited proliferation (82.1%), and long-term treatment demonstrated that the inhibition is dose dependent (46.8 and 85.7%). Interestingly, long-term proliferation of explant tissues at the higher PD concentrations was equally attenuated as the short-term treatment, suggesting a response with PD can be achieved early in treatment. Having defined the proliferative response of explant tissues to PD, additional studies were performed to evaluate the capacity of PD to enhance the therapeutic potential of IR (Supplementary Figure 4B). These *ex vivo* data, similar to the data obtained in Figure 3, indicated that PD has the potential to cooperate with IR. As the data above indicated that the response of PCa cells is dependent on RB, explant tissues were transduced with the shNS and shRB1 constructs and treated with or without PD (Supplementary Figure 4C). As expected, based on the cell and xenograft data, PD reduced the proliferation of explant tissue that had been transduced with shNS. Importantly, PD was ineffective in explant tissues that had been transduced with shRB1; thus, these data are consistent with the notion that RB is a critical determinant for the response to PD in PCa. These studies, using xenografts *in vivo* and human PCa tissues *ex vivo*, demonstrate that CDK4/6

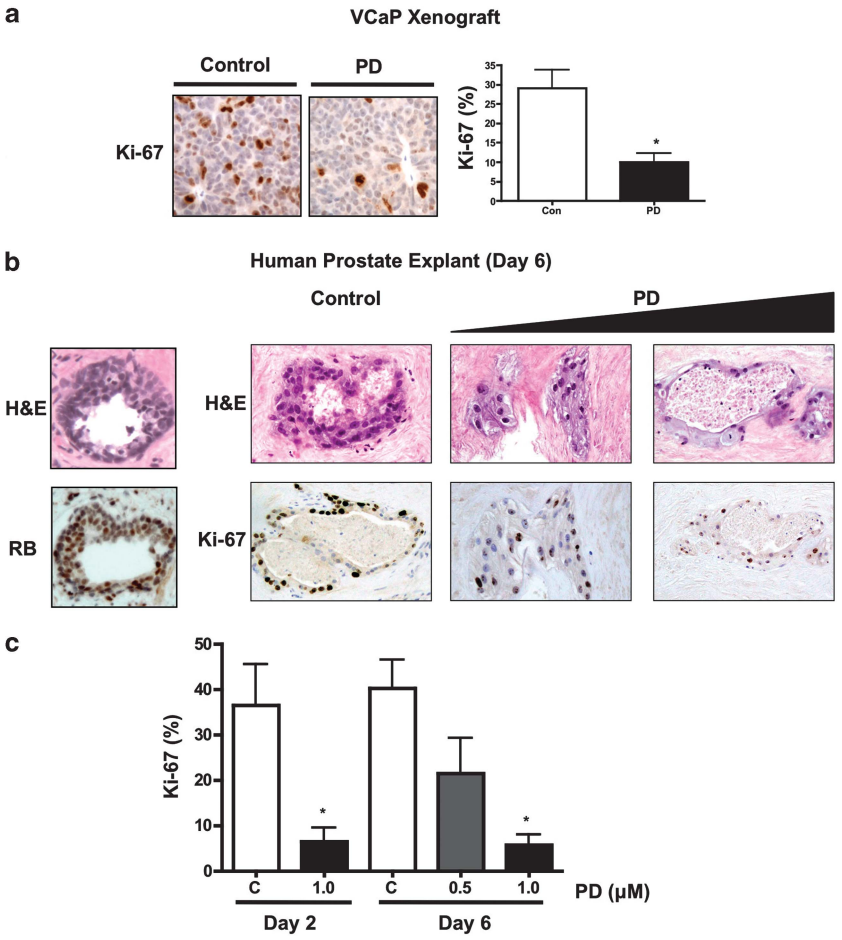

Figure 5. CDK4/6-specific inhibition suppresses proliferation of xenografts *in vivo* and primary human prostate tissue *ex vivo*. The efficacy of PD on PCa was determined by: (**a**) Proliferative Ki-67 marker analysis from VCaP xenografts, grown in SCID mice, treated with control ($n=4$ mice) or PD ($n=4$ mice). Mice were treated with 150 mg/kg of PD, as described detail in the Materials and methods section. Representative images (20 ×) are shown (left panel) and Aperio-based quantification of % Ki-67 (right panel). *Indicates a *P*-value <0.05. (**b**) Representative images from histo- and immunohistological analyses of human prostate tissues used for *ex vivo* tissue explant culture: left panel, images (20 ×) from hematoxylin and eosin (H&E) and RB-stained tissue before processing for culture; right panel, H&E images (20 ×, upper row) and Ki-67 images (20 ×, lower row) from *ex vivo* explant tissues treated 6 days with control (left column) and 0.5 (middle column) or 1 μM PD (right column). (**c**) Aperio-based quantification of % Ki-67 from human explant tissues cultured *ex vivo* for a short (day 2) and long (day 6) treatment with control (C) or 0.5 or 1 μM PD. Bars represent (mean ± s.e.m.) from five patient specimens. *Indicates a *P*-value <0.05.

can be selectively targeted in an RB-dependent manner to limit proliferation and growth. Overall, these data suggest that PD can be developed as an adjuvant means to suppress tumor growth in RB-positive tumors.

DISCUSSION

Proliferation and growth are hallmark phenotypes in cancer and are frequently regulated by the activity of CDK4/6.[7] In the past, therapeutic targeting of CDK4/6 activity has been limited to pan-CDK inhibitors, often resulting in adverse effects.[6] Most notably, for PCa, the broad spectrum CDK inhibitor flavopiridol resulted in adverse effects and toxicity in phase I trials.[10,50] During phase II trials, the dosage was reduced, patients were removed because of unacceptable toxicity, and increased adverse effects were observed, indicating that drug toxicity was a contributing factor for the poor efficacy.[11] Recently, a selective CDK4/6 inhibitor (PD) has been developed with a more favorable phase I toxicity profile;[12,13] however, the impact of PD in PCa has not been explored. Herein, it was demonstrated that PD: (1) effectively limited proliferation of hormone-sensitive and CRPC cells *in vitro*, xenografts *in vivo* and primary human tumor tissues *ex vivo*; (2) cooperated with existing treatments to enhance therapeutic impact without negating standard ADT; and (3) was mechanistically dependent on the RB tumor suppressor, suggesting the necessity for precision medicine. These pre-clinical findings represent the most comprehensive assessment of PD as a viable and potential combination therapy for PCa.

The molecular target of PD is exceedingly clear, in that it is a highly selective CDK inhibitor with a well-documented potency for CDK4/6 (IC_{50} ~10–15 nM) as compared with CDK2 (IC_{50} >5 μM).[12] CDK4/6 activity serves a specialized role, to initiate proliferation of a number of diverse tumor types, through the phosphorylation and inactivation of the RB tumor-suppressor family.[38] In PCa, it has been shown that RB-directed CDK4 activity is important for androgen-dependent proliferation.[3] Importantly, PD-inhibited PCa proliferation/growth and RB phosphorylation at Ser780 (a known CDK4/6 phosphorylation site), providing additional evidence that the mechanism underpinning androgen-dependent proliferation is mediated by CDK4. Interestingly, CDK4 levels are maintained in PCa,[51] suggesting that CDK4 activity has a significant role in proliferation. Androgen withdrawal or stimulation does not alter

CDK4 protein levels, suggesting the possibility of targeting CDK4 in combination with standard ADT. Previous investigation revealed that androgen-stimulated CDK4 activity, in part, is regulated by mammalian target of rapamycin-dependent induction of D-type cyclins.[3] Thus, it would be of future interest to simultaneously target CDK4/6 and mammalian target of rapamycin. It has been recently shown that mammalian target of rapamycin inhibition confers sensitivity of PCa cell proliferation/growth to IR.[44] For patients with locally advanced PCa, addition of radiotherapy has become standard-of-care;[52] therefore, the ability of PD to cooperate with IR in the PCa models tested suggests that CDK4/6 inhibition alone or in combination with other treatments may be beneficial. Consistent with this, others have shown that PD may cooperate with radiation to suppress glioblastoma multiforme tumor cell growth.[32] However, additional studies are needed to reveal the full potential of PD in the context of locally advanced PCa. Overall, these data support a model wherein cyclin D1–CDK4/ are integral to PD-mediated cell cycle inhibition in PCa cells and suggest the feasibility for combinatorial therapy.

In accordance with a cyclin D1–CDK4/6-dependent pathway, PD modestly upregulated cyclin D1 protein levels in the LNCaP and LAPC4 cells. The mechanism of PD-mediated cyclin D1 induction is somewhat enigmatic as therapeutics that result in G1-arrest often exhibit increased cyclin D1 turnover.[40] Initially, it was thought that PD could be inducing a compensatory induction of cyclin D1 expression; however, the levels of cyclin D1 induction were not sufficient to override the PD-mediated cell cycle inhibition. Co-immunoprecipitation analysis of CDK4 indicated a modest increase in cyclin D1 association, suggesting a potential mechanism whereby PD may retain cyclin D1 and CDK4 into an inactive complex, thereby protecting cyclin D1 from turnover. A recent crystallographic study of cyclin D1/CDK4 indicated that CDK4 was in an inactive configuration that resembled other inactive CDKs or p19-inhibited CDK6.[53–56] Although speculative, PD could be inducing a similar inactive conformation of CDK4. Unfortunately, no structural analysis is available for cyclin D1/CDK4 in the presence of PD and low-resolution (3 Å) analysis of cyclin V/CDK6 with PD does not indicate an inactive conformation.[57] Nonetheless, based on the well-characterized ability of cyclin D1, independent of CDK4, to control AR activity, it was hypothesized that PD-induced cyclin D1 accumulation/sequestration with CDK4 might limit the ability of cyclin D1 to hinder genomic AR activity resulting in a modest increase in AR target genes. Consistent with this, AR target gene expression (that is, *KLK3*/PSA) with PD was, in general, modestly increased in the absence or presence of DHT in cells that exhibited increased cyclin D1 (that is, LNCaP and LAPC4). Interestingly, PD exposure in VCaP cells did not show evidence of cyclin D1 accumulation; yet, AR target gene expression was modestly elevated but only in the absence of DHT, suggesting additional factors are involved. One factor may be CDK6 as it has been shown to interact with and enhance AR activity, independent of its kinase activity or association with cyclin D1.[58] In addition, CDK6 overexpression in LNCaP cells displayed increased *KLK3*/PSA expression as well as increased secreted PSA protein in the absence or presence of androgen. Alternatively, although phospho- or total extracellular signal-regulated kinase levels were unchanged by PD (Supplementary Figure 1D), it is possible that PD influences other non-genomic AR targets. Thus, it will be important to discern the broader implications of PD-induced cyclin D1 accumulation with regard to regulating AR activity. It will also be of interest to determine if cyclin D1 and/or CDK6 has any predictive value concerning the response to PD or the potential bypass to therapy in PCa.

As with many drug interventions, a major hurdle is to accurately assess the probability of treatment response or development of resistance. To this point, a number of potential mechanisms have been implicated that may circumvent PD action. For example, elevated cyclin E1 levels could confer downstream CDK2 activation, as has been suggested by gene expression profiling from a panel of PD-resistant ovarian cancer cells.[37] However, cyclin E1 protein levels in PCa cells after PD treatment did not reveal any induction, suggesting that other mechanisms may exist or longer-term treatments are needed to observe changes in potential mechanisms of PD resistance. It is generally held that the majority of CDK4/6 activity targets RB;[38] therefore, based on initial and continuing reports describing PD action, the most probable candidate to nullify the response to PD is RB loss. This study demonstrates that PD-mediated inhibition of PCa proliferation requires RB. Moreover, it was recently identified that disruption of RB is frequently observed in late-stage, human CRPC.[46] These observations suggest that RB disruption might predict the development of resistance to therapeutic agents that inhibit CDK4/6 activity. In addition to allelic loss, RB inactivation occurs through a host of mechanisms that retain RB protein but cripple its tumor-suppressor function.[38] Thus, it is hypothesized that tumors stratified based on gene expression 'signatures' indicative of functional RB could significantly impact therapeutic potential.[59] To this end, we tailored a robust cohort of genes using multiple model systems that reflect functional RB and have validated its application using gene expression data from human PCa and CRPC specimens.[39] Based on the knowledge that RB is generally inactivated during the transition to late-stage CRPC, it is postulated that early-stage PCa patients stratified according to functional RB would benefit from PD-mediated CDK4/6 inhibition.

In this study, a number of well-characterized CRPC cells maintained responsiveness to PD implying that a subset of patients with CRPC may have functional RB and could potentially benefit from targeted CDK4/6 inhibition. Therefore, understanding the timing and response to cell cycle therapy in CRPC is of the utmost importance as the vast majority of patients who succumb to disease have CRPC.[1] In addition, recent observations have shown that CRPC cells have developed unique alterations in cell cycle.[60] It will be of interest to determine if PD alters the 'rewired' cell cycle program in the CRPC setting. Finally, it was shown that CRPC cells were amenable to PD treatment when grown in conditions that mimic standard ADT. Therefore, it will be of interest to examine the combination of PD with more recently developed second-generation, hormone-based therapies[61] (for example, CYP17A1 inhibitors, abiraterone; or AR antagonists, MDV3100) or approved anti-mitotic chemotherapies[62] (for example, microtubule stabilizers, docetaxel) in the context of CRPC as well as hormone-sensitive PCa.

In summary, this study provides a compelling rationale for the use of CDK4/6-selective inhibitors, such as PD, in the treatment of PCa. These pre-clinical and mechanistic findings suggest that CDK4/6 inhibition may provide benefit for PCa patients through: (i) suppressed proliferation/growth in both hormone-sensitive and castration-resistant contexts, (ii) potential combinatorial therapy with IR or hormone-based therapeutics and (iii) approaches that use personalized medicine to evaluate functional RB status before treatment. Together, these studies not only highlight the clinical potential of PD to benefit PCa patient outcome, but also support a role for CDK4/6-specific inhibitors for the treatment of cancers that are dependent on the cyclin/CDK/RB-axis.

MATERIALS AND METHODS

Cell culture and treatments

Androgen-dependent PCa (LNCaP, LAPC4 and VCaP) and CRPC (LNCaP-shRB1, C4-2, 22Rv1 and PC3M) cells were maintained as previously described.[46,63] Unless otherwise indicated, cells ($1.8 \times 10^4/cm^2$) were plated 24 h in growth media using standard serum (5–10% fetal bovine serum) and then treated 24 h with 0.5 μM PD (Pfizer, New York, NY, USA) or 0.1% dimethylsulphoxide. C4-2 and 22Rv1 cells were treated as above, except cells were washed thrice in phosphate-buffered saline and plated in

phenol red-free growth media containing 5% charcoal-dextran-treated serum (HyClone, Thermo Fisher Scientific, Pittsburgh, PA, USA) to mimic castrate conditions.

Flow cytometry

PCa cells were challenged, as described above, and both adherent and non-adherent cells were harvested, gently re-suspended in 100% ethanol, and fixed overnight at −20 °C. Proliferation was measured by bivariate flow cytometry using a 2-h pulse-label of BrdU (Amersham (GE Healthcare Life Sciences), Pittsburgh, PA, USA, RPN201) before harvest and cell cycle position using propidium iodide staining, as described.[44] A Coulter Epics XL Flow Cytometer (Beckman Coulter, Indianapolis, IN, USA) was used to capture 20 000 BrdU/propidium iodide or 15 000 propidium iodide events for proliferation and cell cycle position, respectively. FlowJo software (TreeStar, Ashland, OR, USA) was used to gate for percent BrdU incorporation or cell cycle position using the cell cycle algorithm.

Immunoblotting

Control- and PD-treated cells, described above, were harvested to evaluate protein expression and interaction of cell cycle components. Briefly, total protein (30 μg) was separated by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride and immunoblotted overnight at 4 °C. Antibodies and dilutions (1:1000) used are: AR (SC-816), CDK4 (SC-601); cyclins: A (SC-596), D1 (Neomarkers, Fremont, CA, USA, AB3) and E (SC-198); ppRB-pS780 (Cell Signaling, Beverly, MA, USA, 9307), Ran (BD Biosciences, San Jose, CA, USA, 610340). Immunoblots were quantified using a LI-COR Odyssey (Li-Cor, Lincoln, NE, USA).

Gene expression

To assess AR activity, cells were plated 72 h in phenol red-free growth media containing 5% charcoal dextran-treated serum then treated 24 h with various combinations of DHT (1 nM), Csdx (10 μM) and PD (0.5 μM). RNA was isolated and complementary DNA generated using the Trizol and Superscript methods, respectively. Quantitative PCR was performed for *KLK3*/PSA, *TMPRSS2* and *KLK2* and normalized to *glyceraldehyde 3-phosphate dehydrogenase* using published methodologies.[43]

Irradiation, clonogenic assay

Combinatorial therapy was determined by plating cells (day 0) in growth media containing standard serum conditions and treated with PD (day 1), described above, then 2 Gy of IR using a Pantak X-RAD orthovoltage X-ray irradiator (calibrated daily using a Victoreen dosimeter, Victoreen, Cleveland, OH, USA). Following treatment, growth media (lacking PD) was replaced (days 2 and 5) and cells were counted (days 3, 5 and 7) by a hemacytometer using the Trypan blue exclusion method. Cell doubling times were calculated as described.[64] Briefly, doubling time in days = ((log 2) × ($t_f - t_i$))/(log (q_f/q_i)) where t_i = initial time, t_f = final time, q_i = cell number at t_i, q_f = cell number at t_f. Clonogenic survival was determined by plating LNCaP cells at varying densities in poly-L-lysine coated 50 ml flasks. After 24 h, cells were treated with 0.5 μM PD and IR or IR alone (at indicated IR doses). Cells were allowed to grow for 14 days, colonies were fixed in 100% cold ethanol and stained with 1% crystal violet in 2.5% acetic acid.

Xenografts

Xenograft studies were performed in accordance with NIH Guidelines and animal protocols were approved by Thomas Jefferson University. VCaP cells (1.5×10^6) were combined 1:1 with Matrigel (BD Biosciences, 354234) and inoculated subcutaneously into the flanks of 6 weeks, intact-male mice (NCI-Frederick, Frederick, MD, USA; NOD.SCID/NCr, 01N31). Tumors were measured with calipers and matched for an average size of 700 mm^3, oral gavage was initiated (day 0) with PD (150 mg/kg, $n = 4$ mice) or control (sodium lactate pH 8.0, $n = 4$ mice) and re-dosed twice (days 2 and 4). Tumors from control- and PD-treated mice were harvested (day 5), 24 h after the final treatment. Tumors were processed and sections stained for Ki-67 (1:250; Invitrogen, Carlsbad, CA, USA; 18-0191Z) using described methodology.[65]

Primary human prostate tumor explants

Primary tumor tissue was obtained from patients diagnosed with PCa who underwent radical prostatectomy at Thomas Jefferson University Hospital in accordance with Institutional Review Board standards and in compliance with federal regulations governing research on de-identified specimens and/or clinical data (45 CFR 46.102(f)). Tumors were dissected by a clinical pathologist under sterile conditions and collected in processing media: improved minimum essential medium (5% fetal bovine serum, 0.01 mg/ml insulin (Invitrogen, 12585-014), 30 μM hydrocortisone (Sigma-Aldrich, St Louis, MO, USA, H-0888) and penicillin/streptomycin). Tissue was minced into ~1 mm^3 pieces and placed (2–3 pieces per well) in a 24-well plate on pre-soaked, 1 cm^3 dental sponges (Novartis Animal Health, Greensboro, NC, USA, Vetspon) containing 0.5 ml of processing media with either 0.5 or 1 μM PD. Treatments and controls were refreshed every 48 h and explants were harvested at early and late time points (days 2 and 6, respectively) for histological assessment. Control- and PD-treated explants ($n = 5$ patients) with evidence of glandular epithelial tissue, as determined by a clinical pathologist, were stained for Ki-67 using clinically approved protocols by the Thomas Jefferson University Hospital. Xenograft and explant stained slides were quantified for percent Ki-67 using an AperioScope AT and Spectrum software using the nuclear staining algorithm (Aperio Technologies, Vista, CA, USA).

ABBREVIATIONS

AR, androgen receptor; CDK, cyclin-dependent kinase; CRPC, castration-resistant prostate cancer; Csdx, casodex; DHT, dihydrotestosterone; KLK3/PSA, kallikrein-related peptidase 3/prostate-specific antigen; PCa, prostate cancer; PD, PD-0332991; RB, retinoblastoma.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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